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(54) **PROCEDES ET COMPOSITIONS POUR INHIBER L'ACTIVITE DE LA 5.ALPHA.-REDUCTASE**

(54) **METHODS AND COMPOSITIONS FOR INHIBITING 5.ALPHA.-REDUCTASE ACTIVITY**

(57) L'invention concerne une nouvelle classe de composés anti-androgènes comprenant des acides gras saturés et insaturés, le gallate de catéchine, leurs dérivés et analogues de synthèse, ainsi que leur utilisation pour traiter des troubles associés avec l'activité des androgènes. On décrit également l'utilisation de compositions qui n'étaient pas connues précédemment pour leur activité anti-androgène, pour traiter des cancers et des affections provoquées par l'activité des androgènes. L'invention concerne des procédés pour traiter le cancer du sein, le cancer de la prostate et d'autres tumeurs, au moyen des compositions contenant de la testostérone en combinaison avec des inhibiteurs de la 5.alpha.-réductase tels que des acides gras et des compositions de catéchine. L'invention concerne également des compositions et des procédés pour diminuer la formation de lipides chez un animal, pour diminuer le poids d'un organe d'un individu ou pour diminuer son poids total, traiter les pertes de cheveux, traiter l'hyperplasie bénigne de la prostate, diminuer la sécrétion du sébum et traiter diverses autres affections apparentées.

(57) Disclosed are novel classes of antiandrogenic compounds including saturated and unsaturated fatty acids, catechin gallates, their derivatives, and synthetic analogs, and their use in treating disorders associated with androgenic activities. Also disclosed is the use of compositions not previously known for their antiandrogenic activity in treating disorders related to androgenic activities and cancers. Methods are disclosed for the treatment of breast cancer, prostate cancer, and other tumors using compositions comprising testosterone compositions in combination with 5.alpha.-reductase inhibitors such as fatty acids and catechin compositions. Compositions and methods are also disclosed for use in reducing lipid production in an animal, reducing organ and body weight, treating hair loss, benign prostatic hyperplasia, reducing sebum production, and other related disorders.

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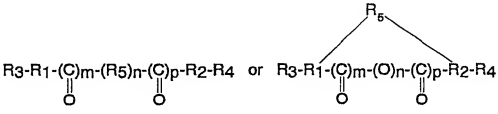
(57) Abstract

Disclosed are novel classes of antiandrogenic compounds including saturated and unsaturated fatty acids, catechin gallates, their derivatives, and synthetic analogs, and their use in treating disorders associated with androgenic activities. Also disclosed is the use of compositions not previously known for their antiandrogenic activity in treating disorders related to androgenic activities and cancers. Methods are disclosed for the treatment of breast cancer, prostate cancer, and other tumors using compositions comprising testosterone compositions in combination with 5α-reductase inhibitors such as fatty acids and catechin compositions. Compositions and methods are also disclosed for use in reducing lipid production in an animal, reducing organ and body weight, treating hair loss, benign prostatic hyperplasia, reducing sebum production, and other related disorders.

CLAIMS

1. A composition comprising a fatty acid or catechin composition and a testosterone composition in a pharmaceutically acceptable vehicle.

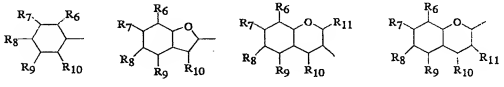
2. The composition of claim 1, wherein said fatty acid is γ-linolenic acid or has the following structure:



wherein m, n, and p is 0 or 1; R_1, R_2, and R_5 have a 0 to 6-atom chain consisting of C, N, S or O, with each of the atoms in the chain having a substitution of -H, -OH, $-CH_3$, $-OCH_3$, $-OC_2H_5$, $-CF_3$, $-CHF_2$, -SH, $-NH_2$, halogen, =O, $-CH(CH_3)_2$ or $-C(CH_3)_3$;

wherein atoms in R_5 are connected to atoms in R_1 and R_2;

wherein R_3 or R_4 are -H, -OH, $-CH_3$, $-OCH_3$, $-OC_2H_5$, $-CF_3$, $-CHF_2$, -SH, $-NH_2$, halogen, =O, $-CH(CH_3)_2$, or $-C(CH_3)_3$, or the following groups:

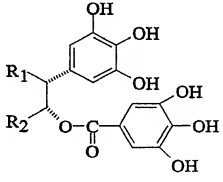


wherein the ring structures may contain unsaturated bond(s) such as in benzen-rings; and

wherein R_6 to R_{10} can be: -H, -OH, $-CH_3$, $-OCH_3$, $-OC_2H_5$, $-CF_3$, $-CHF_2$, -SH, $-NH_2$, halogen, =O, $-CH(CH_3)_2$, $-C(CH_3)_3$, galloyl, or gallolyl groups.

3. The composition of any preceding claim, wherein said testosterone composition is testosterone propionate.

4. The composition of any preceding claim, wherein said catechin has the following structure:



or an isomer thereof, wherein n=1 or 2; R_1 and R_2 are independently H, halogen, lower alkyl, OH, or OR_3; and

wherein R_3 is lower alkyl, or pharmaceutically acceptable salts thereof.

5. The composition of any preceding claim, wherein said catechin is epicatechin, epicatechin gallate, epicatechin-3-gallate, epigallocatechin-3-gallate, (-)epigallocatechin-3-gallate, or (+)epigallocatechin-3-gallate.

6. The composition of any preceding claim, wherein said catechin comprises a composition isolated from green tea, Yunnan tea, gunpowder tea, oolong tea, black tea, Chlorella, black shitake mushroom, basil leaves, parsley leaves, *Angelica sinensis*, *Anisi stellati fructus*, *Codonopsis pilosula*, *Ligustici rhizoma Salvia mitiorrhiza* or Lilly flower.

7. The composition of any preceding claim, for use in inhibiting 5α-reductase activity in a target cell of an animal, wherein the composition is administered to said target cell in an amount effective to inhibit 5α-reductase activity.

8. The composition of claim 7, wherein said target cell is a cancer cell, such as a prostate cancer cell, a breast cancer cell, or a tumor cell.

9. The composition of claim 6, wherein said target cell is located within an androgen sensitive organ such as prostate, preputial organ, ventral prostate gland, dorsolateral prostate gland, coagulating gland or seminal vesicle.

10. The composition of any preceding claim, for use in reducing the weight of an androgen dependent organ in an animal, wherein the composition is administered to said animal in an amount effective to produce a weight decrease in said organ.

11. The composition of claim 10, wherein said androgen dependent organ is a prostate, preputial organ, ventral prostate gland, dorsolateral prostate gland, coagulating gland or seminal vesicle.

12. The composition of any preceding claim, for use in arresting or reducing cancer cell growth in an animal, wherein the composition is administered to said animal in an amount effective to arrest or reduce said cancer cell growth.

13. The composition of claim 12, wherein said cancer cell is a prostate cancer cell, a breast cancer cell, or a tumor cell.

14. The composition of any preceding claim, for use in reducing weight in an animal, wherein the composition is administered to said animal in an amount effective to reduce the weight of said animal.

15. The composition of claim 14, wherein administering said compound to said animal produces a decrease in weight of an androgen sensitive organ such as a prostate gland, coagulating gland, seminal vesicle or a preputial gland.

16. The composition of any preceding claim, for use in inhibiting lipid production in a cell, wherein the composition is administered to said cell in an amount effective to inhibit lipid production.

17. The composition of claim 16, wherein said lipid is sebum.

18. The composition of any preceding claim, for use in reducing hair loss in a human, wherein the composition is administered to said human in an amount effective to reduce hair loss in said human.

19. The composition of any preceding claim, for use in reducing acne in a human, wherein the composition is administered to said human in an amount effective to reduce acne in said human.

20. Use of a composition according to any preceding claim, in the preparation of a medicament for decreasing or inhibiting 5α-reductase activity in a human subject, wherein the medicament is administered to said subject in an amount effective to decrease or inhibit 5α-reductase activity.

DESCRIPTION

METHODS AND COMPOSITIONS FOR INHIBITING 5α-REDUCTASE ACTIVITY

1. BACKGROUND OF THE INVENTION

The present invention is a continuation-in-part of U. S. Serial Number 08/442,055, filed May 16, 1995, which is a continuation-in-part of U.S. Serial Number 07/904,443, filed July 1, 1992, which is a continuation-in-part application of U. S. Serial Number 07/889,589 filed May 27, 1992; the entire text and figures of which disclosures are specifically incorporated herein by reference without disclaimer. The United States government owns certain rights in the present invention pursuant to grants DK41670 and CA58073 from the National Institutes of Health.

1.1 Field of the Invention

The present invention relates generally to compounds, compositions and methods regulating the actions of androgens and other steroid hormones by modulating the activity of 5α-reductase (5-AR). More particularly, the present invention relates to the use of these compounds to treat disorders that are caused by abnormal androgen action in cells or organs. This invention also deals with the use of natural and synthetic fatty acids and catechins, especially polyunsaturated fatty acids and their derivatives and epigallocatechin gallates, as 5-AR inhibitors and as therapeutic agents.

1.2 Description of the Related Art

Uses of androgens known to the medical arts include, for example, treatment of hypogonadism and anemia (Synder, 1984; Mooradian *et al.*, 1987). The abuse of androgen among athletes to enhance performance is well known (Strauss and Yesalis, 1991). Androgens are also known to promote the development of benign prostatic hyperplasia (BPH) (Wilson, 1980), prostate cancer (Huggins and Hodges, 1940), baldness (Hamilton, 1942), acne (Pochi, 1990), hirsutism, and seborrhea (Hammerstein *et al.*, 1983; Moguilewsky and Bouton, 1988). Approximately 70% of males in the United States over the age of 50 have

pathological evidence of BPH (Carter and Coffey, 1990). Prostate cancer is the second leading cause of cancer death in males in the United States (Silverberg and Lubera, 1990; Gittes, 1991). Male-patterned baldness can start as early as the teens in genetically susceptible males, and it has been estimated to be present in 30% of Caucasian males at age 30, 40% of Caucasian males at age 40, and 50% of Caucasian males at age 50. Acne is the most common skin disorder treated by physicians (Pochi, 1990) and affects at least 85% of teenagers. In women, hirsutism is one of the hallmarks of excessive androgen action (Ehrmann and Rosenfield, 1990). The ovaries and the adrenals are the major sources of androgen in women.

1.2.1 Differential Actions of Testosterone and 5α-Dihydrotestosterone (5α-DHT)

In men, the major androgen circulating in the blood is testosterone. About 98% of the testosterone in blood is bound to serum proteins (high affinity binding to sex-steroid binding globulin and low affinity binding to albumin), with only 1-2% in free form (Liao and Fang, 1969). The albumin-bound testosterone, the binding of which is readily reversible, and the free form are considered to be bioavailable, and account for about 50% of total testosterone. Testosterone enters target cells apparently by diffusion. In the prostate, seminal vesicles, skin, and some other target organs it is converted by a NADPH-dependent 5-AR to a more active metabolite, 5α-DHT. 5α-DHT then binds to androgen receptor (AR) in target organs (Anderson and Liao, 1968; Bruchovsky and Wilson, 1968; Liao, 1975). The 5α-DHT-receptor complexes interact with specific portions of the genome to regulate gene activities (Liao *et al.*, 1989). Testosterone appears to bind to the same AR, but it has a lower affinity than 5α-DHT. In tissues such as muscle and testes, where 5-AR activity is low, testosterone may be the more active androgen.

The difference between testosterone and 5α-DHT activity in different androgen-responsive tissues is further suggested by findings in patients with 5-AR deficiency. Males with 5-AR deficiency are born with female-like external genitalia. When they reach puberty, their plasma levels of testosterone are normal or slightly elevated. Their muscle growth accelerates, the penis enlarges, voice deepens, and libido toward females develops. However, their prostates remain non-palpable, they have reduced body hair, and

they do not develop acne or baldness. Females with 5-AR deficiency do not have clinical symptoms (Imperato-McGinley, 1986).

The findings in 5-AR deficient patients suggest that inhibitors of 5-AR would be useful for the treatment of prostatic cancer, BPH, acne, baldness, and female hirsutism. Clinical observations and animal experiments have indicated that spermatogenesis, maintenance of libido, sexual behavior, and feed-back inhibition of gonadotropin secretion do not require the conversion of testosterone to 5α-DHT (Brooks *et al.*, 1982; Blohm *et al.*, 1986; George *et al.*, 1989). This is in contrast to other hormonal therapies which abolish the actions of both testosterone and 5α-DHT.

Treatments of androgen-dependent skin and prostatic diseases by 5-AR inhibitors would be expected to produce fewer side effects than the presently available hormonal therapies. These include castration, estrogen therapy, high doses of superactive gonadotropin-releasing hormone such as Luprolide, and the use of competitive antiandrogens which inhibit AR binding of testosterone and 5α-DHT, such as flutamide, cyproterone acetate and spironolactone. The long term efficacy of 'competitive antiandrogens' is also compromised by their block of the androgenic feedback inhibition of gonadotropin secretion. This results in elevated gonadotropin secretion, which in turn increases testicular secretion of testosterone. The higher level of testosterone eventually overcomes the action of the antiandrogen.

1.2.2 Biological Importance of 5-AR

Excessive 5α-DHT is implicated in certain androgen-dependent pathological conditions including BPH, acne, male-pattern baldness, and female idiopathic hirsutism. It has been shown that 5-AR activity and the 5α-DHT level are higher in the presence of BPH prostates than that of the patients with normal prostates (Isaacs, 1983; Siiteri and Wilson, 1970). 5-AR activity is reported to be higher in hair follicles from the scalp of balding men than that of nonbalding men (Schweikert and Wilson, 1974).

1.2.3 Steroidal 5-AR Inhibitors

The most potent inhibitors of 5-AR developed so far are steroids or their derivatives. Among these the 4-azasteroidal compounds (Merck Co.) are the most extensively studied (Liang *et al.*, 1983; Rasmusson *et al.*, 1986). These inhibitors are 3-oxo-4-aza-5α-steroids with a bulky functional group at the 17β-position, and act by reversibly competing with testosterone for the binding site on the enzyme.

The A-ring conformation of these compounds is thought to be similar to the presumed 3-*enol* transition state of the 5-AR of 3-oxo-Δ^4-steroids. A prototype for 5-AR inhibitors is 17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (4-MA), which behaves as an inhibitor of 5-AR *in vivo*, decreasing the prostatic concentration of 5α-DHT in intact male rats or in castrated male rats given testosterone propionate. 4-MA attenuated the growth of the prostate of castrated rats induced by testosterone, but had much less of an effect in rats given 5α-DHT (Brooks *et al.*, 1981).

When dogs are treated with 4-MA, the prostate size decreases (Brooks *et al.*, 1982; Wenderoth and George, 1983). Topical applications of 4-MA to the scalp of the stamptail macaque, a primate model of human male pattern baldness, also prevented the baldness which normally occurs at puberty in these monkeys (Rittmaster *et al.*, 1987). These results also suggest that the growth of the prostate in rats and dogs, and baldness in the stamptail macaque depend on 5α-DHT. On the other hand, studies in rat pituitary cultures showed that complete inhibition of testosterone conversion to 5α-DHT by 4-MA did not affect testosterone inhibition of LH release, indicating direct action of testosterone in this system (Liang *et al.*, 1984).

Another potent inhibitor is Proscar™ (Merck Co.) (Finasteride, MK-906, or 17β-N-t-butylcarbamoyl-4-aza-5α-androst-1-en-3-one). The inhibitor has no significant affinity for the rat prostate 5-AR. In clinical trials, Proscar™ decreases the plasma level of 5α-DHT and the size of the prostate and also improves urinary flow in patients with benign prostatic hyperplasia (Vermeulen *et al.*, 1989; Rittmaster *et al.*, 1989; Gormley *et al.*, 1990; Imperato-McGinley *et al.*, 1990). In stamptail macaque monkeys, Proscar™ administered orally at 0.5 mg/day, alone or in combination with topical 2% Minoxidil™, reduced serum 5α-DHT level, and reversed the balding process by enhancing

hair regrowth by topical Minoxidil™ (Diani *et al.*, 1992). The effects of Minoxidil™ and Proscar™ were additive.

Among other steroidal compounds shown to inhibit 5-AR are 4-androstane-3-one-17β-carboxylic acid (Voigt *et al.*, 1985), 4-diazo-21-hydroxymethyl-pregnane-3-one (Blohm *et al.*, 1989), and 3-carboxy A-ring aryl steroids (Brandt *et al.*, 1990).

1.2.4 Effects of Fatty Acids and Lipids

Since treatments of androgen-dependent skin and prostatic diseases by 5-AR inhibitors can produce fewer side effects than the hormonal therapies which indiscriminately inhibit all androgen actions, it is desirable to provide different types of 5-AR inhibitors.

Several membrane-associated enzymes (*e.g.*, 5'-nucleotidase, acetyl CoA carboxylase) have been shown to be affected by the polyunsaturated fatty acid content of dietary fat, and to alter the physicochemical properties of cellular membranes (Zuniga *et al.*, 1989; Szepsesi *et al.*, 1989). Various types of phospholipases in rat ventricular myocytes are modulated differentially by different unsaturated fatty acids in the culture media (Nalboone *et al.*, 1990). In addition, treatment of cerebral cortical slices (Baba *et al.*, 1984) or intact retina (Tesoriere *et al.*, 1988) with unsaturated fatty acids can enhance adenyl cyclase activities.

Few studies have been directed to the elucidation of the mode of action of free fatty acids on enzymes in cell-free systems. Certain *cis*-unsaturated fatty acids, at 50 μM, were shown to stimulate protein kinase C activity (Dell and Severson, 1989; Khan *et al.*, 1991) and to inhibit steroid binding to receptors for androgens, estrogens, glucocorticoids, and progestins (Vallette *et al.*, 1988; Kato, 1989). It has not been shown that unsaturated fatty acids can affect steroid receptor binding of steroid hormones *in vivo* in an animal or human.

1.2.5 Prostate Cancer

Prostate cancer is now the most commonly diagnosed cancer in American men. In 1996, 317,100 new prostate cancer cases are expected and 41,400 men may die from prostate (Parker *et al.*, 1996). The growth and development of prostate cancer is initially androgen-dependent, and androgen ablation therapies have been the standard treatment fro metastatic prostate cancer since Charles Huggins published his classic report in 1941 (Huggins and Hodges, 1941). Prostate cancer patients treated with androgen ablation therapy normally have remission of their prostate cancer, but within a few years, tumor regrowth occurs. The recurrence of prostate cancer is largely due to progression of initially androgen-dependent prostate cancer cells to tumor cells that do not depend on androgen for their proliferation (Dawson and Vogelzang (Eds), 1994; Coffey, 1993; Geller, 1993). The reason for this loss of androgen dependency is not known, but human prostate cancer cells, including various LNCaP sublines, have been used to study the changes occurring during progression and tumorigenesis (Kokontis *et al.*, 1994; Thalmann *et al.*, 1994; Joly-Pharaboz *et al.*, 1995; Liao *et al.*, 1995). Some androgen-independent prostate cancer cell lines, such as PC-3 and DU-145, lack androgen receptor (AR) (Tilley *et al.*, 1990). However, AR has been found in metastatic prostate cancer after ablation therapy (Hobisch *et al.*, 1995) and progression to steroid insensitivity can occur irrespective of the presence of functional steroid receptors (Darbre and King, 1987).

2. SUMMARY OF THE INVENTION

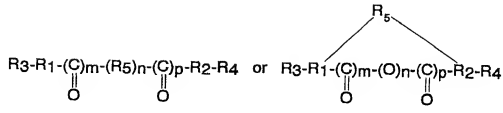
The present invention relates generally to the utilization of certain fatty acids, catechins, and catechin derivatives for the control of androgen activity in target organs and cells through the modulation of 5-AR activity. In certain aspects, particular fatty acids and catechin compounds are employed to repress androgenic activity by inhibiting the formation and availability of active androgen in target cells. Consequently, the invention is useful for the treatment of a wide variety of conditions including, but not limited to, the treatment of prostatic hyperplasia, breast cancer, prostatic cancer, tumor formation, hirsutism, acne, male pattern baldness, seborrhea, and other diseases related to androgen hyperactivity. In another important aspect, several of these compounds have also been shown to effectively decrease

body weight and, in some cases, to decrease the weight of an androgen-dependent body organ, such as the prostate and other organs. The effectiveness of these compounds may be dependent also on their action on other mechanisms involved in angiogenesis, cell-cell interaction, and on their interaction with various components of organs or cells.

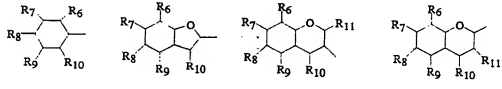
Compounds useful in the practice of the present invention include various isomers of saturated and unsaturated fatty acids, natural and synthetic analogs, and derivatives from which these fatty acids can be generated as well as the metabolite and oxidation products of these fatty acids. The use of these and other fatty acids and their derivatives is also contemplated. Also useful are catechin compounds, particularly, catechins that are structurally similar to epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). EGCG has an additional hydroxyl group on the epicatechin gallate molecule which has been found to be surprisingly active in modulating several 5-AR mediated processes. EGCG derivatives having such an additional OH group on the ECG molecule were shown to be active in inducing body weight loss and particularly in reducing the size of androgen sensitive organs such as preputial glands, ventral prostate, dorsolateral prostate, coagulating glands, seminal vesicles, human prostate tumors, and breast tumors in nude mice.

The inventors have discovered the importance of certain structural features of some catechin compounds which appear to contribute to activity toward 5-AR. The presence of an additional hydroxyl group in gallocatechin gallate as compared with catechin gallate has a significant effect on activity as reflected in the ability to reduce body and organ weight and tumor growth in animals. The structural requirements for activity therefore are EGCG which has one extra -OH group on the ECG molecule was considerably more active than ECG in inducing body weight loss, and in reducing the sizes of preputial gland, ventral prostate, dorsolateral prostate, coagulating glands, seminal vesicles, and tumors of the prostate and breast.

The general formula for 5-AR inhibitors is as shown:



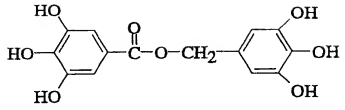
m, n, and p can be 0 or 1. R_1, R_2, and R_5, can have 0 to 6 atom chain consisting of C, N, S or O, and can contain unsaturated bond(s). Each of the atoms in the chain can have a substitution of -H, -OH, $-CH_3$, $-OCH_3$, $-OC_2H_5$, $-CF_3$, $-CHF_2$, -SH, $-NH_2$, halogen, =O, $-CH(CH_3)_2$ or $-C(CH_3)_3$. Atoms in R_5 is connected to atoms in R_1 and R_2. R_3 or R_4 can be: -H, -OH, $-CH_3$, $-OCH_3$, $-OC_2H_5$, $-CF_3$, $-CHF_2$, -SH, $-NH_2$, halogen, =O, $-CH(CH_3)_2$, or $-C(CH_3)_3$, or the following groups:



Where the ring structures may contain unsaturated bond(s) such as in benzen- rings.

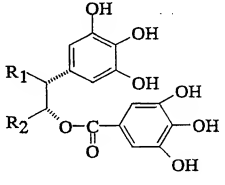
R_6 to R_{10} can be: -H, -OH, $-CH_3$, $-OCH_3$, $-OC_2H_5$, $-CF_3$, $-CHF_2$, -SH, $-NH_2$, halogen, =O, $-CH(CH_3)_2$, $-C(CH_3)_3$, galloyl, or galloyl groups.

Carbon-carbon linkages in R_1 to R_{11} may be saturated or have double bonds. A carbonyl group may be in an enol form. For example, the following compound has been found to be potent inhibitor of 5-AR:



The active compounds may include ester linkages that may be hydrolyzed to the active unsaturated fatty acids, catechins, or the structure shown. In addition, R1 and R2 need not be individual substituents, but may represent together aromatic or heterocyclic moieties and containing halogen or alkyl substituents. Alternatively, R1/R2 may represent alicyclic moieties with one or more isolated double bonds. Combining all of the information obtained, the structures shown above comprise a group of novel 5-AR inhibitors.

For catechin gallates and their derivatives, the following general structure is noted:



The fatty acid and catechin compounds are believed to effect the transformation of androgens by inhibition of 5-AR and, as a result, (a) limit the supply of dihydroxytesterone (5α-DHT) to target organs and suppress the 5α-DHT dependent androgen actions, and/or (b) prevent the metabolic loss of testosterone or other androgenic precursors of 5α-DHT and promote or maintain hormone actions that are dependent on testosterone or other 5α-DHT precursors. These compounds may act by controlling organogenesis, angiogenesis and/or cellular interaction with other chemical agents.

Steroids other than testosterone or dihydroxytestosterone are also substrates of 5-AR. It is expected, therefore, that the fatty acid and catechin compounds disclosed herein will also regulate the transformation and activation of other 3-oxodelta^4-steroids and therefore control the biological functions of other steroid hormones through the same mechanism. An advantage to the use of fatty acid and catechin compounds of the present invention, and particularly some of their derivatives, is their relative stability *in vivo* and *in vitro*. In general, one may prepare derivatives that are not easily metabolized, degraded or incorporated into lipid structures or other derivatives. Stability may, for example, be increased by alkylation, cyclization, fluorination, *etc.* One will of course not wish to prepare derivatives that interfere with the functional aspects of the fatty acid or catechin. Certain of the fatty acid and catechin compounds are particularly effective in exhibiting organ specificity without significant side effects and in such cases one would choose to prepare derivatives that would not significantly increase side effects.

As discussed, there are certain structural features of the catechins that contribute to their utility and effectiveness in particular uses, such as inhibition of sebum production. This appears also to be true for the selection of fatty acids that are active inhibitors of 5-AR. As shown in Tables 1 and 2, a relatively large number of polyunsaturated fatty acids inhibit 5-AR activity. While the glycerides, esters, nitriles and chlorides showed little activity in the cell binding assays, some of these compounds are likely to be hydrolyzed outside the cells, or hydrolyzed after entering cells, to form the free fatty acid. In comparison with free fatty acids, it may be desirable to administer free fatty acids as glycerides or other derivatives that are relatively more stable to oxidation and/or are less readily metabolized than the free acids. Such derivatives are, of course, considered to be therapeutically active compounds.

In general, the inventors have observed that where fatty acid compounds are employed for inhibition of 5-AR activity, the length of the fatty acid carbon chain, as well as the position and number of double bonds in the molecules, appear to relate to activity. The highest activities are observed with 14 or more carbon atoms and at least one, preferable two or more, double bonds. The effectiveness of the unsaturated fatty acids is dependent on the positions of double bonds in the carbon chains.

In addition to certain fatty acids, it has been shown that gallates of catechins and gallocatechins are effective 5-AR inhibitors. This class of inhibitors includes a relatively large group of related compounds, some of which have been isolated and identified. These compounds are found in several types of plant bark and leaves, particularly tea and, most particularly, in green tea. Catechins with galloyl substitution showed surprising activity as inhibitors of 5-AR. These compounds include catechin gallate (CG), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), the optical isomers, and conjugated substances such as theaflavins and theaflavin mono- (or di-) gallates. The latter compounds are components of fermented teas including black tea.

The inventors determined that active catechin gallates have three distinct groups in their molecules: (a) a 3-flavonol substituent; (b) a 3′, 4′, 5′-trihydroxybenzen (gallolyl) group attached to the 2-position of the flavonol; and (c) a gallic acid that forms an ester linkage (galloyl) with the 3-OH of the flavonol. The three groups may independently contribute to the inhibitory action, but the effect on 5-AR appears to be synergistic. Certain synthetic gallate derivatives (such as methyl gallate and *n*-propyl gallate, 3,4,5-trihydroxybenzamide, gallic acid and pyrogallol) were not as active as catechin gallate, indicating that the gallolyl or galloyl structure alone was not sufficient for high inhibitory activity. A low inhibitory activity was found within octyl gallate indicating that for the inhibitory activity, the flavonol group of catechin gallates may be replaced by other groups having similar geometric structures. Based on the lower activities of catechin or epicatechin compared with their gallate derivatives, it appears that the essential structural feature required for high 5-AR inhibition is an acyl (galloyl) or a trihydroxybenzen group that forms an ester or ether linkage with the flavonol.

By analogy with the fatty acid compounds, the inventors expect that certain active catechin gallates may not enter target cells easily. However, esterification of hydroxyl groups on the inhibitory compounds should enhance the ability of these compounds to enter the target cells. Once inside the cells, esters would be readily hydrolyzed by esterases to alcohols (*e.g.,* epigallocatechin gallate) that can inhibit 5-ARs (Williams, 1985).

In another aspect of the invention, γ-LA was found to be a particularly potent 5-AR inhibitor. The ability of γ-LA to inhibit 5-AR in solubilized microsomes indicates that

the γ-LA inhibition may not be rigidly dependent on the native source of endoplasmic reticulum membranes. The fatty acid inhibitor may act by interacting with the reductase and/or other components that are vital for reductase activity. The inhibitory fatty acids may also interact with and potentiate other endogenous inhibitors or interfere with lipids which may potentiate the reductase. A proposed mechanism (Brandt *et al.*, 1990) for the 5-AR (E) reaction includes the following steps:

$$\text{NADPH} + \text{E} \longleftrightarrow [\text{NADPH-E}] \xleftarrow{+\text{Testosterone (T)}} (\text{A}) \longrightarrow [\text{NADPH-E-T}] \longrightarrow [\text{NADP}^+\text{-E-5}\alpha\text{-DHT}] \xrightarrow{+\text{H}^+} (\text{b}) \longrightarrow 5\alpha\text{-DHT} + [\text{NADP}^+\text{-E}] \rightarrow \text{NADP}^+ + \text{E}$$

It was surprising that two *trans* isomers of fatty acids, *i.e.*, elaidic acid and linolelaidic acid, had little inhibitory activity in the [^{3}H]4-MA binding assay, yet were as potent as their cis-isomers, oleic and linoleic acid, in the enzymatic assay. The *cis*-unsaturated fatty acids may inhibit the formation of [NADPH-E-T] (step a); whereas the trans isomers act at points after the formation of the ternary complex (step b).

In certain embodiments the disclosed methods are useful for reducing weight in an androgen dependent organ. The inventors have demonstrated that certain fatty acids and catechins are effective in reducing the weight of androgen dependent organs, including the preputial gland, ventral prostate, dorsolateral prostate, seminal vesicles, coagulating gland, and at high doses, also the testes. This effect was observed with several fatty acids. The most effective correlated with those that showed the most inhibitory activity toward 5-AR. In a preferred embodiment, γ-LA was shown to be particularly effective in reducing the weight of androgen dependent organs; in particular, the ventral, prostate and preputial organ. It is evident that a relatively broad range of long chain polyunsaturated fatty acids will have the desired effect in reducing the weight of androgen dependent organs. One will select such fatty acids based on, for example, *in vivo* stability, ease of administration, and release in active form. Certain ester or ether derivatives are expected to be hydrolyzed by cell esterases to an active form; for example, glycerides. A particularly preferred long chain polyunsaturated fatty acid is γ-LA. This fatty acid, as well as related derivatives and

compounds, are particularly effective. Contemplated derivatives are esters, particularly hydrolyzable esters.

The invention also includes the inhibition of 5-AR in cells by contacting the cells with a composition comprising at least one catechin compound. Several catechins including (-)epicatechin gallate (ECG) and (-)epigallocatechin gallate (EGCG) reduced the weight of the androgen dependent organs, ventral prostate and preputial organ; however, EGCG also reduced body weight by as much as 35% in some case, suggesting a potential use of this compound and related species as weight loss agents. EGCG would be ideal for weight loss programs because of its lack of toxicity or apparent side effects. EGCG and related catechins occur naturally in several types of plants, including tea, and thus have a long history of safety as a component of a food item.

EGCG, EGC and γ-LA are particular examples of catechins and fatty acids that reduce weight of androgen-sensitive organs. The inventors believe that these compounds reduce lipid or sebum production in male hormone sensitive organs, for example, in ventral and dorsal lateral prostate glands, coagulating glands, and seminal vesicles. EGCG and ECG are structurally similar in that EGCG has eight hydroxyl groups compared with the seven hydroxyl groups in EGC, yet EGCGC is significantly more effective than ECG in promoting weight reduction. The effect of EGCG on lipid production or organ weights may be dependent on a specific EGCG interaction with a macromolecule that is specific for EGCG on the modulation of cell-cell or protein-protein interactions, or regulation of enzyme activity or gene expression. Regulation or modulation of the interaction or the function of the EGCG receptor or protein complex by natural or synthetic compounds would be expected to offer a means to control the lipid synthesis or the growth and function of androgen-sensitive organs.

In more particular aspects of the invention, the inventors have discovered that certain catechins, particularly EGCG, can be administered to promote body weight loss that differentially affects overall body weight and prostate weight loss. In particular examples, it was shown that for a certain percentage of overall body weight loss, prostate weight loss was percentage-wise more than three times as much. The loss in body weight and the organ weight are likely due to EGCG interference of a common step in the pathway

controlling body and organ weight gain. EGCG and related compounds may interact and interfere with a receptor macromolecule (probably containing a protein) that modulates specific lipid synthesis or accumulation. Lipids can modulate gene expression, cell development and differentiation, and organ growth. Specific interference of lipid metabolism in the cells and organs may control the growth of the organs, in particular, prostate sebaceus organs, preputial organs and other secretory organs. In certain applications, it is expected that benign or abnormal growth or cancer of these organs may be treated or even prevented by administration of catechin related compounds.

It has been demonstrated that catechin compounds will arrest or reduce human prostate and breast cancer cell growth. The effectiveness of catechin compounds was shown by the inventors to be dependent on the methods these compounds were administered to the experimental animals. The inventors found that intraperitoneal application was much more effective than oral route. It is expected that direct application to the prostate having tumor will be very effective. The inventors demonstrated that EGCG was surprisingly effective in suppressing and even reducing the size of human prostate and breast tumors in animal models. The effect was illustrated with EGCG; however, structurally similar catechin compounds should also be effective, particularly those that are structurally similar to EGCG in having at least one additional hydroxyl group as compared with EGC. Thus, the EGCG species that contains eight hydroxyl groups is significantly more effective in reducing body weight than is EGC, which contains seven hydroxyl groups. Compounds of this general structure are expected to be particularly effective in chemoprevention and chemotherapy of human prostate cancer. Compounds having a part of structure similar to a part of structure of EGCG are also expected to be effective also. In an important aspect of the invention, the inventors have demonstrated that effective treatment of prostate and other cancers may be accomplished using pharmaceutical compositions comprising a novel combination of a testosterone composition, such as testosterone propionate and one or more of the catechins and/or fatty acids disclosed herein found to be effective in decreasing prostate tumor size in an animal. These novel testosterone and fatty acid/catechin compositions have been shown to surprisingly be useful in the treatment of these and other cancers. It is contemplated that the testosterone employed in formulation of the composition may include

various other salts and derivatives of testosterone, and as such, are all contemplated to be useful and fall within the scope of the present disclosure. These novel compositions find use in the treatment methods also disclosed and claimed herewith.

For determining the efficacy of the compositions described herein, the inventors have demonstrated a useful animal model for quantitating lipid production is the rat skin model. In rat sebaceous glands, as in human, sebum lipids are synthesized in the intermediate cells by the smooth endoplasmic reticulum (SER). The volume density of SER, as seen under electron microscopic examination, depends on androgen (Moguilewsky and Bouton, 1988). Since repression of androgen action can cause reduction of this density, the effectiveness of test compounds, systemically or topically administered to rats, can be evaluated by measuring their ability to reduce the volume density of SER.

The fatty acids and catechin compositions disclosed herein can be used as antiandrogenic agents through topical or systemic application. A preparation for this purpose can include a carrier, a protectant, an antioxidant (such as vitamin C or E and various catechins and polyphenols), and other pharmaceutical and pharmacological agents. In preferred embodiments, a further component of the composition is a testosterone composition and/or one or more catechin composition. It is also expected that such fatty acids can be used in a delivery system involving molecular recognition through which the said fatty acids are delivered to target sites. Such a delivery system may involve, among other methods, liposome techniques or immunological devices.

Natural or synthetic chemicals that can modulate the production or cellular action of receptors and macromolecules may be useful in the treatment of abnormalities such as obesity, BPH, prostate cancer, skin diseases, baldness, breast cancer, a variety of tumors, and hirsutism, which are related to lipid synthesis, body weight, and/or androgen function.

The inventors have used that animal models to demonstrate the effectiveness of the disclosed compositions, and particularly the fatty acids and catechin compounds such as EGCG and related compounds on a variety of cancers. For example, Shionogi tumor and other tumor induced tumors have been studied in male rats. Human breast and prostate cancer cell growth are studied in nude mice. Alternatively, rodent breast tumors induced by

carcinogens and other cancers induced in transgenic mice or Dunning tumors in rats are also analyzed for their chemotherapy by the catechin and γ-LA composition disclosed herein.

Other aspects of the invention include methods for screening inhibitors of sebum production. While other animal models may be used, the inventors have found it convenient to use humans for screening. The method basically involves applying a compound suspected of inhibiting sebum production to some portion of the human body on the skin area that has sebaceus glands. These areas include the human forehead, as well as other areas of faces and hands. Ideally, the applications will cover two bilaterally similar areas, with one area designated a control area and the other a test area. One will then measure sebum production in each of these areas. Several ways of measuring sebum production may be employed; however, a convenient means is to use a clear tape over each area for a specified length of time. This length of time is conveniently 30-40 minutes, but could be shorter or longer; *e.g.* 10 minutes or 2-3 or more hours. Longer periods of time, however, will result in generally more sebum production and would be employed only in cases where sebum production is low or difficult to obtain. The use of a clear tape is particularly convenient because each tape may then be removed from the subject and the amount of sebum deposited on the tape measured or determined by such means as light scattering, decrease in light transmission, *etc.*

The inventors have found that regardless of the measurement means employed, it is rapid and convenient to assign a relative and arbitrary value for sebum production to each measurement. Use of arbitrary values avoids the necessity of absolute measurements and outside control samples because the control area tape may be used as a relative control. It has been found that when the ratio of the value for the test area to that of control is lower than the ratio before the application of the test compound to the control area, the test compound is a suitable candidate for use in sebum suppression. When identified by this method of screening, compounds that exhibit a lower ratio will be useful as topical agents.

The use of the fatty acid and catechin compounds disclosed in the present invention, in therapeutically effective amounts of pharmaceutical compositions containing one or more of the compounds of the invention, in some cases in combination with other therapeutic agents and carriers, or in natural or synthetic products, is appropriate in the treatment of

various disorders. In particular embodiments, such as in the treatment of prostate tumors and prostatic cancer, the inventors have demonstrated the inclusion of testosterone in the pharmaceutical preparation surprisingly increases the effectiveness of these compounds in treating the particular disorder, and to reduce the extent or spread of the cancer. These disorders include, but not necessarily limited to, those conditions wherein excessive androgenic activities have been implicated, for example, male pattern baldness, female hirsutism, acne, BPH, and cancers (including tumors) of prostate, breast, skin and other organs.

These pharmaceutical compositions, comprising certain fatty acids, catechins, catechin derivatives, either alone or in combination with testosterone compositions, can be administered by topical or internal routes, including oral, injection, or other means, such as topical creams, lotions, hair tonics, scalp care products, or transdermic patch applications, alone or in combination with other compounds of the invention and or with other drugs, drug additives, or pharmaceutical compounds. Combination of unsaturated fatty acids and catechins will be beneficial for clinical or cosmetic treatments because they individually may selectively control the activities of different enzymes or isozymes, and they may act to stabilize each other or protect active compound from degradation or alteration by chemical, biological or environmental condition during the preparation, application or storage of the compounds or products. It has been demonstrated that some of these compounds appear to regulate steroid metabolism, and may thereby affect the function of normal or mutated hormone receptors. Therefore, these compositions are useful in the treatment of androgen and other hormone-sensitive or insensitive disorders or tumors. The compounds of the invention are also important in the studies of the mechanism of action of hormones and anti-hormones.

When oral routes of administration are contemplated, the catechin compositions may be ingested in their original form, *i.e.* herbs, food, tea, *etc.* either directly or as extracts of the various natural products which contain the catechin compounds. In certain embodiments it may be desirable to administer the compositions in combination with vitamins or antixoidants and/or GLA or the like. When administration of the catechin compositions with

testosterone is desired, the testosterone may be co-administered by any of the standards methods, such as *i.v., i.p., subq.,* or orally as desired.

As used herein the terms "contact", "contacted", and "contacting", are used to describe the process by which an effective amount of a pharmacological agent, *e.g.,* an inhibitor of 5-AR, comes in direct juxtaposition with the target cell. As used herein the term "cell" refers to cells capable of fatty acid synthesis. What is meant by an effective amount is the amount of drug necessary to give the therapeutically desired level of 5-AR inhibition.

Although the present invention has been described primarily in terms of its clinical usefulness, as indicated by the art accepted model of inhibition of sebum production used in the practice of the present invention, the methods and compositions herein will also be useful in methods for screening a candidate substance for 5-AR stimulatory properties in combination with compositions of the present invention. Such a method would comprise preparation of different isozyme of 5-AR including isozymes genetically engineered and expressed in cells; obtaining a candidate substance; contacting a culture of sebaceous cells with said candidate substance; simultaneously contacting said culture with a composition of the present invention having 5-AR inhibitory activity; and determining the extent of 5-AR inhibition. 5-AR inhibition using compositions of the present invention may also be utilized in such methods to provide a baseline control for determining the efficacy of a candidate substance, as well as to test such a candidate substance for synergistically enhancing the 5-AR inhibitory activity of the compositions disclosed herein. As used herein, a "candidate substance" is defined as any substance or compound, either naturally occurring or synthetic that is suspected to affect 5-AR activity.

3. BRIEF DESCRIPTION OF THE DRAWINGS

The drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. General formula for compounds that are part of the present disclosure. R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, or R_9 may be a hydrogen, a fluorine or other halogen, or a methyl, ethyl, propyl, other alkyl or aryl group; one or two fluorine or other halogen atom(s) may replace hydrogen attached to any carbon atom(s) and 'l', 'm', 'n', 'p', 'q', 'r', and 't' are each independently 0 or an integer from 1 to about 50 and, preferably from 1 to about 30. The alkyl or aryl group and fluorine or other halogens attached to the molecules may protect them from degradation by oxidation of the unsaturated double bonds and α, β or ω oxidation. Oxidation products and metabolites of these fatty acids are also included since they are also expected to regulate 5-AR activity. Also -CH and the -OH groups can be in a substituted form (-CR and/or -OR) wherein -R represents an alkyl or an aryl group. Also included are acylates and esters that, upon hydrolysis, can form the carboxylic acid shown. 'X' can be a carbon, a sulfur, an oxygen, or a -NH-. This X-linkage is not limited to link carbon 2 and the carbon at the end of the chain; the link can be between any two carbons in the carbon chain. For protection of a fatty acid from oxidative degradation, it may be useful to incorporate one or two sulfur atoms into the backbone carbon chains. The total carbon chain length can be 6 to 28.

FIG. 2. Fatty acids which can be used to regulate 5-AR activity.

FIG. 3. Examples of fluorinated and cyclic derivatives of fatty acids that are part of the present disclosure.

FIG. 4A. General structure of catechin derivatives.

FIG. 4B. Structure of galloyl moiety.

FIG. 5. Structure of important catechins.

FIG. 6. Structure of important catechin gallates.

FIG. 7. Novel classes of 5-AR inhibitors. R1 and R2 are alkyl, allyl, or groups having general structures of VII, VIII, IX, X, XI, XII. R3 and R4 are groups having general structures of XI or XII. R5 and R6 are hydrogen or halogen atoms. R7, R8, R9, R10, R11, R12, R13, R14, R15, and R16 are hydrogen, halogen, hydroxyl, methyl, ethyl, methoxyl, acetyl, or acetoxyl group. R is oxygen, nitrogen, or sulfur atom.

FIG. 8. γ-LA inhibition of forehead sebum production in a human male.

FIG. 9. Catechin inhibition of forehead sebum production in a human male.

FIG. 10. Stimulation of hamster flank organ by topical application of testosterone (T) and dihydrotestosterone (5α-DHT). The right flank organs of immature castrated male hamsters (5 each group) were treated topically with 5 μl/day of ethanol solution alone (C), or ethanol containing 0.5 μg T or 5α-DHT for 17 days. One representative animal from each group is shown.

FIG. 11. Effect of testosterone (T) stimulation on the application site (right flank organ) versus the contralateral site (left flank organ). The right flank organ of immature castrated male hamsters was treated with T (0.5 μg/day) for 17 days. The left flank organ was not treated.

FIG. 12. Inhibition of testosterone-stimulated growth of the pigmented macule of the hamster flank organ by γ-LA, but not by stearic acid. Male hamsters (4 weeks old) were castrated and treatment was started 2 weeks later for 18 days. The animals were treated with 5 μl of ethanol (C), ethanol containing testosterone (T, 0.5 μg), T (0.5 μg) + γ-LA (LA, 1 mg), T (0.5 μ g) + stearic acid (SA, 1 mg), or T (0.5 μg) + SA (2 mg). Only the right flank organ was treated and shown here. The data collected from these animals are shown in Table 7.

FIG. 13. γ-LA applied to the right flank organ of intact male hamster produced a localized inhibition of the growth of the pigmented macule stimulated by endogenous androgens. The right flank organs of intact male hamsters, 4 weeks old, were treated for 156 days as described in Table 7. The treatment consisted of topical solutions of vehicle (ethanol) alone (C) or γ-LA (1 mg/flank organ/day). The fight flank organs of 2 representative hamsters are shown.

FIG. 14. γ-LA topically applied to the right flank organ of intact hamsters inhibited only the application site and not the contralateral (left) flank organ. A representative intact hamster treated with 1 mg γ-LA from group 3 of the study described in Table 7 is shown.

FIG. 15. γ-LA reduced the pigmentation and the length of the hair in the flank organ. The hamsters shown here are the same animals shown in Table 7. The picture represents the hair growth on the flank organ during the last two days. The hair of the flank organ of the group treated with 1 mg γ-LA/5 μl ethanol/day (A) was markedly lighter in color and shorter in length than the vehicle (C) treated hamsters.

FIG. 16. The effect of γ-LA treatment on the growth rate of the pigmented macules from intact male hamsters. The right flank organs of intact prepubertal male hamsters, 4 weeks old, were treated topically with vehicle alone (control), γ-LA 1 mg or 2 mg/5 μl ethanol/flank organ/day. There were 10 animals per treatment group. The index of the area of the pigmented macules was determined at the beginning (Day 0) and after various days of treatment. The left flank organs received vehicle only. The growth rates of the pigmented macules of the left flank organs of all 3 groups were similar to that of the right flank organ of the control group.

FIG. 17. Tumor size suppression by EGCG in nude mice.

FIG. 18. Reduction of tumor size in nude mice following EGCG therapy.

FIG. 19. Effects of ECG and EGCG on hamster ventral prostate size.

FIG. 20. Effects of ECG and EGCG on hamster preputial gland size.

FIG. 21. Effects of EC, ECG, EGC and EGCG on body weight gain in rats.

FIG. 22. Restoration of normal body weight gain following cessation of EGC and EGCG treatment in rats.

FIG. 23. Androgen-specific suppression of the growth of LNCaP 104-R2 tumors in castrated male nude mice. Nude mice were castrated and injected with LNCaP 104-R2 cells. Four weeks later, mice with tumors (240 ± 20 mm^3) were implanted with a 20 mg pellet of testosterone (T), testosterone propionate (TP), 5α-dihydrotestosterone (5α-DHT), 5β-dihydrostestosterone (5β-DHT), medroxyprogesterone (MPA), or 17 β-estradiol (E2). Tumor size was measured every week. Each point represents data for 6 to 15 tumors. Control mice were castrated but did not receive a steroid pellet implant.

FIG. 24. Stimulation of the growth of LNCaP 104-S tumors by testosterone propionate (TP) in castrated male nude mice. LNCaP 104-S cells were injected into normal male nude mice and 4 weeks later, nude mice bearing tumors (260 ± 20 mm^3), were castrated and divided into 2 groups. One group received no additional treatment (C), and mice in the other group were implanted with testosterone propionate (C + TP). Tumor size was measured every week. Each point represents data for 5 tumors.

FIG. 25. Testosterone-dependent suppression and finasteride-dependent stimulation of the growth of LNCaP 104-R2 tumors in castrated male nude mice. Castrated nude mice were injected with LNCaP 104-R2 cells and after 4 weeks some mice received TP implants. After an additional 3 weeks mice not treated with TP (883 ± 63 mm^3) were divided into 2 groups: one was implanted with TP (■) and the other without implants served as controls (○). Mice treated with testosterone propionate (TP) starting at week 4 had small tumors

(88 ± 13 mm^3) and were divided into 3 groups: mice with TP implanted initially at the 4th week (see FIG. 1) and no additional treatment (●), mice implanted with TP at the 4th week and implanted with finasteride (FS) at the 7th week (▲), and mice implanted with TP at the 4th week and removal of implanted TP at the 7th week (□). Tumor size was measured every week. Each point represents data for 5 tumors.

FIG. 2B. The effect of testosterone and finasteride on growth of LNCaP 104-R2 tumors in castrated male nude mice. Representative mice from the study summarized in FIG. 3. A: a castrated nude mouse 7 weeks after injection of LNCaP 104-R2 cells; B: a mouse with LNCaP 104-R2 tumor as in A and implanted with TP at the 7th week and picture taken 1 week later; C: the mouse in B, 3 weeks later; D: a mouse as in A but implanted with TP at the 4th week and picture taken at the 7th week; E: the mouse in D from which TP was removed at the 7th week and picture taken 4 weeks later; F: a mouse, treated as the one shown in D and implanted with finasteride at the 7th week and picture taken 4 weeks later.

FIG. 27. Effect of finasteride on the growth of LNCaP 104-S and MCF-7 tumors in nude mice. Human prostate cancer LNCaP 104-S cells or human breast cancer MCF-7 cells were injected into normal male or female nude mice respectively. After tumors grew to $1,400 \pm 430$ mm^3, nude mice were divided into two groups. One group received finasteride (FS) implants while the other group was kept as control. Each point represent data for 4 tumors.

FIG. 28A. Histology of and immunocytochemical localization of androgen receptor and prostate specific antigen (PSA) in LNCaP tumors. Hematoxylin and eosin stained tissue section for LNCaP 104-R2 tumor from castrated male nude mouse.

FIG. 28B. LNCaP 104-R2 tumor from castrated male nude mouse 1 week after implantation of testosterone propionate.

FIG. 28C. LNCaP 104-R2 tumor from a mouse 4 weeks after testosterone propionate implantation.

FIG. 28D. Immunocytochemical staining (peroxidase-diaminobenzidine) for androgen receptor (D) in a LNCaP 104-R2 tumor from a castrated male nude mouse.

FIG. 28E. PSA in the LNCaP 104-R2 tumor from a nude mouse implanted with testosterone propionate for 1 week.

FIG. 29. The effect of testosterone propionate (TP) on the expression of mRNAs for AR, c-*myc*, PSA and β_2-R2 tumors. The autoradiogram from the RNase protection assay using ^{32}P-labeled antisense probes is shown in inset. The graphic represents quantitation of protected probes normalized to the amount of protected β_2-MG probe. LNCaP 104-R2 cells were injected into castrated male nude mice and 7 weeks after injection of cells, TP was implanted and tumors resected 0, 3, 7, 14 days later for RNA extraction and RNase protection assay.

4. DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

4.1 Steroid Hormones and 5-AR Activity

4.1.1 Androgens

Androgens are one of the six major classes of steroid hormones. Steroid hormones form complexes with specific receptor proteins in selective cells of target organs (Jensen *et al.*, 1968; Liao, 1975; Gorski, *et al.*, 1976). Steroid receptors are members of a superfamily of transcription factors that can regulate gene expression, and this function is dependent on the binding of a specific hormonal ligand to an appropriate receptor (Evans, 1989; Beato, 1989; O'Malley, 1990).

Studies of the specificity and affinity of steroid hormones for their receptors have contributed greatly to the understanding of the relationships among steroid and receptor structures and biological activity, target organ specificity, and the mechanism of action of many antihormones, including "competitive antiandrogens". "Competitive antiandrogens" are

defined herein as those antiandrogens that interact with receptors and competitively prevent receptor binding of active androgens (Fang and Liao, 1969; Liao *et al.*, 1973; Liao *et al.*, 1974; Chang and Liao, 1987; Liao *et al.* 1989), although it should be noted that some compounds with an antiandrogenic activity may act by a different mechanism.

Androgens, produced in the testis, stimulate the differentiation of the male reproductive organs, including the penis, scrotum, prostate, seminal vesicles, epididymis, and vas deferens. With the onset of puberty, an increase in the production of androgen promotes the growth of these tissues. Androgen is required for spermatogenesis and accelerates skeletal muscular growth and bone formation. In the central nervous system, it stimulates libido and produces feedback inhibition of gonadotropin secretion. In skin, androgen increases the size of sebaceous glands and apocrine glands and converts villus hairs in the axillae, pubic region, and the beard to form coarser and longer terminal hairs. Androgen causes thickened vocal cords and lowers the pitch of the voice. Androgen also stimulates hematopoiesis.

Androgen action in many organs, such as prostate is dependent on the conversion of testosterone by a NADPH-dependent 5-AR to 5α-DHT, which then binds to 5-AR to exert its biological function (Liao *et al.*, 1989). The inhibition of 5-AR limits the availability of 5α-DHT but not testosterone, therefore, 5-AR inhibitors are useful in selective treatment of 5α-DHT-dependent abnormalities, such as benign prostate hyperplasia, prostate cancer, hirsutism, male pattern alopecia and acne, without affecting testosterone-dependent testicular function, sexual behavior, and muscle growth (Russell and Wilson, 1994; Hipakka and Liao, 1995). Most 5-AR inhibitors are steroids or compounds with steroid-like structures. The present invention, however, also has identified specific fatty acids and catechins, including γ-LA and EGCG, which are potent 5-AR inhibitors.

It is known that polyunsaturated fatty acids can correct the effects of fatty acid deficiencies that manifest as dermatitis, kidney necrosis, infertility, and cardiovascular diseases (Herold and Kinsella, 1986; Phillipson *et al.*, 1985; Ziboh and Miller, 1990) and also can exhibit anti-tumor activities (Begin, 1990; Karmali *et al.*, 1984). Many unsaturated fatty acids are essential components of mammalian membranes, typically in the acylated form of triglycerides and phospholipids (Lands, 1965).

Arachidonic acid serves as a specific precursor in the biosynthesis of prostaglandins and leukotrienes (Needleman *et al.*, 1986). These metabolites of unsaturated fatty acids are mediators of inflammation. Unsaturated essential fatty acids have been implicated as dietary factors that influence acne. However, no firm support for this view has developed, and no successful treatment based on this idea has appeared (Downing *et al.*, 1986). Synthetic retinoids and AR binding competitive antiandrogens have been used to obtain therapeutic improvement of acne in some individuals. These anti-acne agents increase the proportion of linoleic acid in sebum in parallel with clinical improvement (Wright, 1989).

4.1.2 5-AR

Selective inhibitors of the different types of 5-AR, therefore, are desirable for studies of androgen action and for therapy for androgen-dependent tumors and other abnormalities.

Two 5-AR isozymes have been demonstrated in rats and humans. In the human, type 1 and 2 isozymes have only 50% amino acid sequence homology (Anderson *et al.*, 1991). Type 1 isozyme has a neutral to basic pH optimum and is rather insensitive to the 5-AR inhibitor finasteride. Type 2 isozyme has an acidic pH optimum and is 30 times more sensitive to finasteride inhibition than type 1 isozyme. In the prostate, type 2 isozyme is the major form (Anderson *et al.*, 1991), whereas, in the scalp, type 1 isozyme predominates (Harris *et al.*, 1992). In the rat, it has been shown that the liver contains mainly type 1 isozyme (Berman and Russell, 1993); however, the prostate contains both type 1 (60%) and type 2 (40%) isozymes (Normington and Russell, 1992). γ-LA was found to inhibit 5-AR activity in both the liver and prostate (Liang and Liao, 1992). γ-LA therefore, is an inhibitor of both type 1 and type 2 isozymes. 5-AR isozymes in the hamster flank organ have not been characterized at the molecular level. However, 5-AR activity in hamster flank has an optimum of pH 8 (Takayasu and Adachi, 1972), indicating that the major 5-AR isozyme in the flank organ may be type 1, rather than the type 2 isozyme.

In a given individual, 5-AR activity is found to be higher in balding skin than from hairy skin (Bingham and Shaw, 1973). Some idiopathic hirsute women have a normal circulating level of testosterone, but their affected skin has a higher 5-AR activity than that

of nonhirsute women (Serafini and Lobo, 1985). An increased 5-AR activity has also been reported for skin with acne (Sansone and Reisner, 1971).

Genetic evidence also supports the suggestion that 5α-DHT plays an important role in the development of BPH and the above skin conditions. In males with hereditary 5-AR deficiency, their prostates remain small or nonpalpable after puberty. They do not develop acne, temporal hairline recession, or baldness. Compared to their fathers and brothers, they have scanty beards and reduced body hair.

4.2 Fatty Acid Metabolism

Fatty acids fluorinated at α, β, and ω positions (Gershan and Parmegiani, 1967; Pattison and Buchanan, 1964; Gent and Ho, 1978) and ω-oleic acids (Tosaki and Hearse, 1988) have been identified in plants and microorganisms, and have been chemically synthesized. Many of these fluorinated acids are toxic. Degradation of some fluorinated fatty acids can yield fluoro-acetic acid, which can be incorporated into fluorocitrate and can then block aconitase action. This can cause inhibition of the citric acid cycle and cellular energy production (Hall, 1972). Fluorinated fatty acids are often useful in the studies of fatty acid degradation, metabolism and transport in biological systems (Stoll *et al.*, 1991), and biophysical studies of protein-lipid interaction and membranes functions (Gent *et al.*, 1981).

Biotin is a cofactor of major carboxylases which are necessary for orderly production and metabolism of fatty acids. Alopecia caused by biotin-deficiency can be completely treated by biotin administration to patients. Oral administration and cutaneous application of unsaturated fatty acids can also improve biotin-dependent dermatological conditions including scalp hair growth (Munnich *et al.*, 1980; Mock *et al.*, 1985). The fatty acid effect is apparently due to supplementation of the deficient fatty acids and not related to regulation of androgen action involved in male pattern-alopecia.

4.3 Pharmaceutical Compositions

Aqueous compositions of the present invention comprise an effective amount of the 5-AR inhibitory agent dissolved or dispersed in a pharmaceutically acceptable aqueous medium. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

The preparation of an aqueous composition that contains such an inhibitory compound as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

The pharmaceutical compositions disclosed herein may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or they may be compressed into tablets, or they may be formulated for control release, such as transdermic and osmotic pressure devices, injectable devices and implantable devices, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The percentage of the compositions and preparations may, of course, be varied and may conveniently be 100% (application of pure compounds). The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For

instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

The active compounds may also be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated

above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus ny additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

For oral administration the composition may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

The composition can be formulated in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

In other embodiments, one may desire a topical application of compositions disclosed herein. Such compositions may be formulated in creams, lotions, solutions, or in solid form depending upon the particular application. The formulation of pharmaceutically acceptable vehicles for topical administration is well known of skill in the art (see *i.e.*, "Remington's Pharmaceuticals Sciences" 15th edition). Variation of the dosage of the compositions disclosed herein, will necessarily depend upon the particular subject, and the nature of the condition(s) being treated.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

4.4 Assays For Candidate Substances

In still further embodiments, the present invention concerns a method for identifying new agents that act to inhibit the activity of 5-AR. Those new agents may be termed as "candidate substances." Different types of 5-AR isozymes have been found to be present in different combinations in different cells of various organs (Russel and Wilson, 1994).

Therefore, it is desirable to have isozyme-selective inhibitors for therapeutic purposes. For the sources of type 1 and type 2 5-AR Rat 1A cells were genetically engineered to certain only type 1 or type 2 isozyme. Rat 1A cells or microsomes were used for the screening of isozyme-selective inhibitors. It is contemplated that this screening technique will prove useful in the general identification of any compound that will serve the purpose of inhibiting the activity of 5-AR or specific types of 5-AR. It is further contemplated that useful compounds in this regard will in no way be limited to the specific compositions disclosed herein, but any analogs, derivatives, synthetic modifications, or substitutions of constituents of those compositions which can effectively inhibit this activity either *in vitro* or *in vivo*.

Accordingly, in screening assays to identify pharmaceutical agents which inhibit 5-AR activity, it is proposed that compounds isolated from natural sources such as plants, animals or even sources such as marine, forest or soil samples, may be assayed for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived from chemical compositions or man-made compounds.

The active compounds may include fragments or parts of naturally-occurring compounds or may be only found as active combinations of known compounds which are otherwise inactive.

4.5 Methods of Inhibiting 5-AR Activity

In still further embodiments, the present invention is concerned with a method of inhibiting 5-AR which includes subjecting a cell to an effective concentration of a 5-AR inhibitor such as one of the family of fatty acid or catechin compounds disclosed herein, or with a candidate substance identified in accordance with the candidate screening assay embodiments. This is, of course, an important aspect of the invention in that it is believed that by inhibiting the activity of 5-AR, one will be enabled to treat various aspects of disease and cancers, such as prostate-related cancers and diseases caused by abnormal androgen actions. It is believed that the use of such inhibitors to block abnormal androgen action will serve to treat cancers and diseases and may be useful by themselves or in conjunction with other anti-cancer therapies, including chemotherapy, resection, radiation

therapy, and the like. The compounds of this invention, besides acting as 5-AR inhibitor, may have other effects that can lead to antitumor activity or to suppress abnormal growth of prostate or other organs.

5. EXAMPLES

The following examples illustrate the rationale and practice of the invention. Although many of the examples are based on the actions of androgens and ARs, they may also apply to the function of other steroid hormones which is dependent on or regulated by 5-AR or their isozymes. They are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 Example 1 -- Inhibition of 5-AR Activity

5.1.1 Assays

In mammalian cells, 5-AR is very tightly associated with intracellular membranes, including the membrane of the endoplasmic reticulum and contiguous nuclear membranes. Attempts to solubilize and purify active 5-AR have not been very successful. The assay of 5-AR activity, therefore, has been performed by measuring the rate of conversion of testosterone to 5α-DHT by whole cells or by microsomal and nuclear preparations in the presence of NADPH (enzymatic assay). Alternatively, the 5-AR activity can be reliably assayed by following NADPH-dependent noncovalent binding of a potent radioactive inhibitor, such as [^{3}H]4-MA ([^{3}H]4-MA-binding assay), which strongly competes with testosterone for binding to the reductase. The results of the two assays correlate very well when microsomal preparations from different organs or animals are used for comparison (Liang *et al.*, 1983).

5.1.1.1 [^{3}H]4-MA Binding Assay for 5-AR

The procedure was described in detail previously (Liang *et al.*, 1983, 1990). Briefly, the binding assay solution, in a final volume of 0.15 ml, contained microsomes (2-20 μg of protein), 0.08 μCi of [^{3}H]4-MA, 0.1 mM-NADPH, 1 mM-dithiothreitol and 50 mM-potassium phosphate, pH 7.0, with or without the indicated amount of a lipid or an inhibitor preparation. Lipids were dissolved in ethanol and added in 1-5 μl volumes. Control tubes received the same amount of ethanol. After incubation at 0°C for 1 h, the [^{3}H]4-MA bound to microsomes was determined by collecting microsomes on a Whatman GF/IF glass fibre filter and washing with 10 ml of 20 mM-potassium phosphate, pH 7.0, containing 0.01% CHAPS to remove unbound [^{3}H]4-MA.

5.1.1.2 Assay of the Enzymatic Activity of Microsomal 5-AR

The standard reaction mixture, in a final volume of 0.15 ml, contained microsomes (1 μCi of [^{3}H]testosterone, 0.5-3.0 μM non-radioactive testosterone, 0.1mM-NADPH, 1 mM dithiothreitol and 50 mM-potassium phosphate, pH 7.0, with or without the indicated amount of a lipid or an inhibitor preparation. The reaction was started by the addition of microsomes and the incubation was carried out at 37°C for 15 min. Steroids were extracted and separated by thin layer chromatography as described previously (Liang and Heiss, 1981; Liang *et al.*, 1984a, 1985a). Radioactive steroids were located by fluorography and the amount of radioactivity present was determined by scintillation counting. The 5-AR activity was measured by analyzing the extent of the conversion of [^{3}H]T to [^{3}H]5α-DHT.

5.1.2 Sources of 5-AR Activity

Microsomes were prepared at 4°C from a buffered 0.32 M sucrose homogenate of human liver and from the livers of adult Sprague-Dawley female rats by differential centrifugation as described previously (Liang *et al.*, 1990), and were used in the assay of 5-AR activity. In some experiments, microsomes were solubilized with 0.1% polyoxyethylene ether W-1 as described previously (Liang *et al.*, 1990), except for the substitution of polyoxyethylene ether W-1 for Lubrol®-WX.

Cells genetically engineered to express specific types of 5-AR isozymes may also be used as sources of 5-AR activity. Intact cells containing 5-AR, their microsomes, or nuclear preparation may also be used to screen 5-AR inhibitors.

5.1.3 Inhibitors of 5-AR Activity

Animal and plant sources were tested for the presence of compounds affecting 5-AR activity. Inhibitory activities were found in extracts of rat and beef liver microsomes, beef kidney, human placenta, rat and human prostate as well as in yeast and vegetable plant oils, *e.g.*, corn, peanut and olive oils, indicating the presence of 5-AR inhibitors in a wide range of sources including animal, plant and microorganisms.

5.1.3.1 Rat Liver Microsomes

When the microsomal fraction of rat liver was solubilized with acetic acid and then mixed with methanol, more than 80% of microsomal proteins were removed as precipitates. This procedure inactivated the 5-AR activity completely. The soluble fraction, but not the precipitated fraction, contained compounds that inhibited 5-AR activity (determined by the enzymatic assay or [^{3}H]4-MA-binding assay) of rat liver microsomes. Sephadex® G-50 column chromatography of the methanol soluble fraction showed separation of the inhibitory activity from the majority of the protein peak which eluted in the void volume. The inhibitory activity was also found in methylene chloride extracts of rat liver microsomes, suggesting that some of the inhibitors were lipids.

5.1.3.2 Plants and Fungi

Preparations were also obtained and specific compounds were isolated from various plant materials. Some of these were able to regulate both the type 1 and type 2 5-AR isozymes of rat and human. While some of these agents were inhibitory, other agents stimulated 5-AR activities.

Each plant material (1 to 2 g) was extracted by 2 to 10 ml of water, ethanol, isopropyl alcohol, ether, chloroform, or ethyl acetate. Organic solvents can contain 0-90% of water and the extraction can be carried out at 0-100°C for 30 minutes to 20 hours.

When 1 g of the plant material was extracted with 4 ml of ethanol or water, and 3 μl of the extract was tested in the liver microsomal 5-AR assay system (the final volume of the reaction mixture was 0.15 ml), a significant inhibitory activity (over 20% inhibition) was observed with extracts from various brands of green tea, Yunnan tea, special gunpowder tea, oolong tea, black tea, chlorella, black shiitake mushroom, basil leaves, parsley leaves, and Chinese herbs, including *Angelica sinensis*, *Anisi stellati fructus*, *Codonopsis pilosula*, *Ligustici rhizoma*, *Salvia mitiorrhiza*, and Golden Lilly flower, seeds of borage, evening primrose, black current, sesame, pumpkin, sunflower, and wheat germ.

The inhibitory substances in basil, oolong tea, green tea, and *Angelica sinensis*, could be separated from other inactive substances by one or two dimensional silica gel thin-layer chromatography or by Sephadex™ gel column chromatography. The chemical structures of some purified compounds were determined by comparing their chemical properties with that of standard compounds, including chromatographic mobility, melting point, ultra-violet and visible-light spectra and NMR. Commercially-available standard compounds were also used in 5-AR assays to show that some of them were indeed 5-AR inhibitors that inhibit the formation of 5α-DHT.

5.1.3.3 Fatty Acids

Certain long chain fatty acids and in particular, γ-LA were found to inhibit 5-AR activity. In general, it was found that long chain polyunsaturated fatty acids were most effective, particularly those with at least two double bonds and with a chain length of at least 12.

5.1.3.4 Catechins and Epicatechin Gallates

The major inhibitory substances in various brands of tea preparations, especially in green tea, were found to be catechin derivatives (FIG. 20A). Catechins without a galloyl (FIG. 20B) substitution (FIG. 21) were much less active than catechin gallate, epicatechin gallate, epigallocatechin gallate, and their optical isomers (FIG. 22) or their conjugated substances such as theaflavins and theaflavin mono- (or di-) gallates. These gallates showed significant inhibitory activities (30 to 90% inhibition) at concentrations of 0.5 to 40

μM in the assay systems containing (a) rat liver microsomal preparations or (b) cells infected with retrovirus containing genes for type 1 or type 2 5-ARs and expressing specific type of the reductases. Catechin and epicatechin (FIG. 21) were much less active (less than 25% inhibition at 40 μM).

Although these inhibitory polyphenolic substances are antioxidants, they did not significantly oxidize NADPH under the assay conditions (in the presence of liver microsomal preparation and in the absence of testosterone or 4-MA, indicating that the inhibitory activity was due to the inhibition of 5-ARs and not due to a nonspecific oxidation of NADPH by these polyphenols.

Various synthetic gallate derivatives (methyl gallate, *n*-propyl gallate, 3,4,5-trihydroxybenzamide), gallic acid, and pyrogallol were not as active as catechin gallate. This indicated that the gallol or galloyl structure alone was not sufficient for the high inhibitory activity. A low inhibitory activity was found with *n*-octyl gallate, indicating that, for the inhibitory activity, the flavonol group of catechin gallates may be replaced by other alcoholic group having similar geometric structures.

The results indicated that the gallate moiety incorporating an acyl (galloyl) or alcoholic (trihydroxybenzyl) group may be required for inhibition of 5-AR. These groups may form an ester or ether linkage with the flavonol (FIG. 23).

5.2 Example 2 -- Fatty Acid Inhibition of 5-AR Activity

Identification of compounds that inhibited 5-AR utilized two types of assays; an enzymatic assay and a binding assay as described in Example 1. Both assays identified similar activities for the active fatty acids.

When various lipids were tested for their ability to affect binding of [^{3}H]4-MA to rat liver microsomes, only certain unsaturated fatty acids were inhibitory, as shown in Tables 1 and 2. Among the lipids tested, the highly inhibitory fatty acids have 14 to 22 carbon chains and one to six double bonds. The presence of a double bond was required for higher inhibitory activity; saturated fatty acids were generally not as active as corresponding unsaturated fatty acids. With the [^{3}H]4-MA binding assay, only compounds with double bonds in the *cis* configuration were active at low concentrations (< 10 μM), whereas the

trans isomers were inactive even at high concentrations (> 0.2 mM). However, as is shown in Example 3, the *trans* isomers were active inhibitors when the reductase activity was analyzed using the enzyme assay. The difference in the effect of *cis* and *trans* isomers of fatty acids in the [^{3}H]4-MA binding assay is obvious when the following sets of fatty acids are compared: oleic acid (C18:1, *cis*-9) *vs.* elaidic acid (C18:1, *trans*-9) and linoleic acid (C18:2, *cis*-9,12) *vs.* linolelaidic acid (C18:2, *trans*-9,12). The results presented in Tables 1 and 2 also demonstrate that the number and the position of the double bonds also affected the potency. When the [^{3}H]4-MA binding assay was used, the inhibitory potency for the C18 fatty acids were, in decreasing order: γ-LA (*cis*-6,9,12) > *cis*-6,9,12,15-octadeca-tetraenoic acid > α-linolenic acid (α-LA) (*cis*-9,12,15) > linoleic acid (*cis*-9,12) > oleic acid (*cis*-9) > petroselinic acid (*cis*-6). Erucidic acid (C22:1, *cis*-13) was inactive; whereas *cis*-4,7,10, 13,16,19-docosahexaenoic acid was a potent inhibitor. Undecylenic acid (C11:1,10) and nervonic acid (C24:1, *cis*-15) were also inactive.

A free carboxyl group is important since the methyl ester and alcohol analogs of these inhibitory unsaturated fatty acids were either inactive or only slightly active. Prostaglandin E2, F2a and I2 were not active; whereas the prostaglandin A1, A2, B1, B2, D2, E1, and F1a were somewhat active at 0.2 mM. Carotenes, retinals, and retinoic acid were also inactive. Phosphatidylcholine, phosphatidyl ethanolamine, 3-diolein, retinol, 13-*cis*-retinoic acid, and 13-*cis*-retinol were slightly stimulatory.

When the inhibitory effects of fatty acids were tested by the enzymatic assay, the relative potency of saturated and *cis*-unsaturated fatty acids were in agreement with that obtained by the [^{3}H]4-MA-binding assay (Tables 1 and 2), regardless of whether rat liver microsomes or prostate microsomes were used as the source of the enzyme. The *trans* isomers, elaidic acid (C18:1, *trans*-9) and linolelaidic acid (C18:2, *trans*-9,12) were much less inhibitory than their *cis* isomers, oleic acid (C18:1, *cis*-9) and linoleic acid (C18:2, *cis* 9,12), in the [^{3}H]4-MA binding assay (Tables 1 and 2); however, they were as potent as their *cis* isomers in the enzymatic assay using either prostate microsomes or liver microsomes. The results suggested that the *trans* isomers inhibited 5-AR through a different mechanism.

TABLE 1

Inhibition of [^{3}H]4-MA Binding to 5-AR of Rat Liver Microsomes by Lipids

Test compounds	Numeric symbol #	% Inhibition of [^{3}H]4-MA binding* Concentration of test compounds		
		10 μM	40 μM	200 μM
Control (no addition)				
Undecylenic acid	C11:1 (10)		NA	13±2
Myristoleic acid	C14:1 (*cis*-9)	NA	25±4	43±1
Palmitic acid	C16:0			NA
Palmitoleic acid	C16:1 (*cis*-9)	NA	16±5	73±7
Palmitoleic acid methyl ester			NA	NA
Palmitoleyl alcohol			NA	16±4
Stearic acid	C18:0	NA	NA	NA
Petroselinic acid	C18:1 (*cis*-6)		NA	52±9
Oleic acid	C18:1 (*cis*-9)	NA	16±6	63±12
Elaidic acid	C18:1 (*trans*-9)	NA	NA	NA
Oleic acid methyl ester			NA	NA

(Table 1, continued)

Test compounds	Numeric symbol #	% Inhibition of [^{3}H]4-MA binding* Concentration of test compounds		
		10 μM	40 μM	200 μM
Oleyl alcohol			NA	NA
Linoleic acid	C18:2 (*cis*-9,12)	NA	12±3	86±4
Linolelaidic acid	C18:2 (*trans*-9,12)		NA	19±5
Linoleic acid methyl ester			NA	NA
Linoleyl alcohol		NA	NA	25±5
α-LA	C18:3 (*cis*-9,12,15)	19±3	27±7	84±6
α-LA methyl ester		NA	NA	NA
α-Linolenyl alcohol		NA	NA	24±1
γ-LA	C18:3 (*cis*-6,9,12)	50±2	83±12	96±2
Octadecatetraenoic acid	C18:4 (*cis*-6,9,12,15)	NA	40±6	88±2
Arachidonic acid	C20:4 (*cis*-5,8,11,14)	NA	30±10	88±5
Docosahexaenoic acid	C22:6 (*cis*-4,7,10,13,16,19)	NA	27±1	87±6
Erucic acid	C22:1 (*cis*-13)		NA	NA
Nervonic acid	C24:1 (*cis*-15)		NA	NA

(Table 1, continued)

*Lipids were tested at concentrations ranged from 0.01 to 0.2 mM. Each study was carried out in duplicates and several experiments were performed to assure that the results shown are representative. Compounds that showed less than 10% inhibition were considered not active (NA).

At 200 μM, no significant effect was observed with:

(a) saturated aliphatic fatty acids including caproic acid, heptanoic acid, caprylic acid, nonanoic acid, capric acid, undecanoic acid, lauric acid, tridecanoic acid, myristic acid, pentadecanoic acid, nonadecanoic acid, arachidic acid, heneicosanoic acid, behenic acid, tricosanoic acid, and lignoceric acid,

(b) fatty acyl esters and alcohols including stearic acid methyl ester, S-stearoyl CoA, palmitic acid methyl ester, S-palmitoyl CoA, *cis*-9-tetradecenol, and arachidonyl alcohol, and

(c) vitamin A related compounds including α- and β-carotenes, retinoic acid, 9-*cis*-retinal, retinal, and 13-*cis*-retinal. At this high concentration, some aliphatic lipids showed inhibitory activities that were significantly lower than the corresponding unsaturated fatty acids (percent inhibition in the parentheses): myristoleic acid methyl ester (27%), γ-LA methyl ester (32%), and *cis*-4,7,10,13,,16,19- docosahexenol (51%). Retinol, 13-*cis* retinoic acid, and 13-*cis*-retinol showed 58% stimulation at 200 μM but no stimulation or inhibition at 40 μM. IC50 (the concentrations needed to show 50% inhibition) for potent fatty acids were: γ-LA (10 μM), octadecatetraenoic acid (57 μM), γ-LA (60 μM), arachidonic acid (65 μM), palmitoleic acid (108 μM), linoleic acid (117 μM), and oleic acid (128 μM).

The numeric symbol indicates the number of carbon atoms and double bonds in the molecule. The numbers in parentheses indicate the position of double bonds (numbered from the carboxyl end) in *cis*- or *trans*-forms.

In addition to the compounds shown in Table 1, the fatty acids, their methyl esters and glycerides shown in Table 2 were tested. The carbon chain length of these fatty acids ranged from 11 to 24 carbons with one to 6 double bonds. Some of the inhibitory compounds and the concentrations required for 50% inhibition (shown in parenthesis; NA indicates not inhibitory at 200 μM or lower concentrations) are: 10-pentadecenoic acid (100 μM), 10-heptadecenoic acid (28 μM), 10-trans-heptadecenoic acid (NA), methyl 10-heptadecenoate (NA), 13-octadecenoic acid (93 μM), 12-octadecenoic acid (NA), 11-octadecenoic acid (26 μM), monogamma linolenin (86 μM), γ-linolenyl alcohol (NA), γ-linolenyl acetate (NA), methyl γ-linolenate (NA), cholesteryl γ-linolenate (NA), di-γ-linolenin (NA), γ-linolenoyl chloride (NA), tri-γ-linolenin (NA), 6,9,12,15-octadecatetraenoic acid (74 μM), nonadecane nitrile (NA), 12-nonadecenoic acid (90 μM), 10-nonadecenoic acid (130 μM), 10-trans nonadecenoic acid (NA), 10,13-nonadecadienoic acid (86 μM), linoleyl cyanide nitrile (NA), linolelaidyl cyanide nitrile (NA), 11 eicosenoic acid (146 μM), 8-eicosenoic acid (48 μM), 5-eicosenoic acid (NA), 11,14 eicosadienoic acid (131 μM), trans 11,14-eicosadienoic acid (NA), methyl 11,14 eicosadienoate (NA), 11,14-eicosadienoyl chloride (NA), 11,14,17-eicosatrienoic acid (29 μM), 11,14,17-eicosatrienoyl chloride (NA), 8,11,14-eicosatrienoic acid (15 μM), homo-γ-linolenoyl chloride (NA), methyl homo-γ-linolenate (NA), 5,8,11-eicosatrienoic acid (50 μM), archidoyl chloride (NA), heneicosenoic acid (154 μM), heneicosene nitrile (NA), erucic acid (NA), 13,16-docosadienoic acid (118 μM), 13,16,19-docosatrienoic acid (163 μM), methyl 13,16, 19-docosatrienoate (NA), 7,10,13,16-docosatetraenoic acid (46 μM), methyl docosatetraenoate (NA), 4,7,10, 13, 16, 19-docosahexaenoic acid (47 μM), 14 tricosenoic acid (NA), 15-tetracosanoic acid (NA).

TABLE 2

Inhibition of [^{3}H]4-MA Binding to 5-AR of Rat Liver Microsomes by Lipids

Test compounds	Numeric symbol #	% Inhibition of [^{3}H]4-MA binding* Concentration of test compounds			
		5 μM	10 μM	40 μM	200 μM
Control (no addition)					
Undecylenic Acid	C11:1 (*cis*-10)		NA	NA	13
Myristoleic Acid	C14:1 (*cis*-9)	NA	NA	25	43
10-Pentadecenoic Acid	C15:1 (*cis*-10)	NA	NA	NA	71
Palmitic Acid	C16:0				NA
Palmitoleic Acid	C16:1 (*cis*-9)		NA	16	73
Palmitelaidic Acid	C16:1 (*trans*-9)				NA
10-*cis*-Heptadecenoic Acid	C17:1 (*cis*-10)	NA	NA	83	84
10-*trans*-Heptadecenoic Acid	C17:1 (*trans*-10)	NA	NA	NA	40
Stearic Acid	C18:0		NA	NA	NA
11-Octadecenoic Acid	C18:1 (*cis*-11)	NA	14	55	81
12-Octadecenoic Acid	C18:1 (*cis*-12)	NA	NA	NA	NA

(Table 2 continued)

Test compounds	Numeric symbol #	% Inhibition of [^{3}H]4-MA binding* Concentration of test compounds			
		5 μM	10 μM	40 μM	200 μM
13-Octadecenoic Acid	C18:1 (*cis*-13)	NA	NA	NA	57
trans-Vaccenic Acid	C18:1 (*trans*-11)				39
Oleic Acid	C18:1 (*cis*-9)	NA	NA	16	63
Elaidic Acid	C18:1 (*trans*-9)		NA	NA	NA
Petroselinic Acid	C18:1 (*cis*-6)			NA	52
Linoleic Acid	C18:2 (*cis*-9,12)		NA	12	86
Linolelaidic Acid	C18:2 (*trans*-9,12)		NA	NA	19
α-LA	C18:3 (*cis*-9,12,15)	NA	19	27	84
Linolenoyl Chloride	Chloride				NA
γ-LA	C18:3 (*cis*-6,9,12)	30	50	83	96
Mono-γ-Linolenin	monoglyceride	NA	NA	35	87
γ-Linolenyl Alcohol	alcohol	NA	NA	NA	41
γ-Linolenyl Acetate	acetate	NA	NA	NA	27
Di-γ-Linolenin	Diglyceride		NA	NA	NA
γ-Linolenoyl Chloride	chloride		NA	NA	NA

(Table 2 continued)

Test compounds	Numeric symbol #	% Inhibition of [^{3}H]4-MA binding* Concentration of test compounds			
		5 μM	10 μM	40 μM	200 μM
Tri-γ-Linolenin	triglyceride	NA	NA	NA	NA
6,9,12,15-Octadecatetraenoic Acid	C18:4 (*cis*-6,9,12,15)		NA	40	88
10-*cis*-Nonadecenoic Acid	C19:1 (*cis*-10)	NA	NA	13	79
10-*trans*-Nonadecenoic Acid	C19:1 (*trans*-10)	NA	NA	NA	32
12-*cis*-Nonadecenoic Acid	C19:1 (*cis*-12)	NA	NA	32	91
10,13-Nonadecadienoic Acid	C19:2 (*cis*-10,13)	NA	NA	37	83
5-Eicosenoic Acid	C20:1 (*cis*-5)	NA	NA	NA	NA
8-Eicosenoic Acid	C20:1 (*cis*-8)	14	41	52	81
11-Eicosenoic Acid	C20:1 (*cis*-11)	NA	NA	15	76
11,14-*cis*-Eicosadienoic Acid	C20:2 (*cis*-11,14)	NA	NA	NA	89
11,14-*trans*-Eicosadienoic Acid	C20:2 (*trans*-11,14)	NA	NA	NA	NA
11,14-*cis*-Eicosadienoate	methyl ester	NA	NA	NA	NA
11,14-*cis*-Eicosaidienoyl Chloride	chloride	NA	NA	NA	NA
11,14,17-*cis*-Eicosatrienoic Acid	C20:3 (*cis*-11,14,17)	NA	1	78	94
11,14,17-*cis*-Eicosatrienoyl Chloride	chloride	NA	NA	NA	NA

(Table 2 continued)

Test compounds	Numeric symbol #	% Inhibition of [^{3}H]4-MA binding*			
		Concentration of test compounds			
		5 μM	10 μM	40 μM	200 μM
8,11,14-*cis*-Eicosatrienoic Acid	C20:3 (*cis*-8,11,14)	NA	42	92	82
Arachidonic Acid	C20:4 (*cis*-5,8,11,14)		NA	30	88
Arachidoyl Chloride	chloride		NA	NA	NA
Heneicosenoic Acid	C21:1 (*cis*-12)	NA	15	25	60
Erucic Acid	C22:1 (*cis*-13)		NA	NA	NA
13,16-Docosadienoic Acid	C22:2 (*cis*-13,16)	NA	NA	21	93
13,16,19-Docosatrienoic Acid	C22:3 (*cis*-13,16,19)	NA	NA	NA	65
7,10,13,16-Docosatetraenoic Acid	C22:4 (*cis*-7,10,13,16)	NA	NA	41	79
4,7,10,13,16,19-Docosahexenoic Acid	C22:6 (*cis*-4,7,10,13,16,19)	NA	18	49	86
14-Tricosenoic Acid	C23:1 (*cis*-14)	NA	NA	NA	36
15-Tetracosenoic Acid	C24:1 (*cis*-15)			NA	NA

5.3 Example 3 -- γ-LA Inhibition of 5-AR

γ-LA is one of the more potent inhibitors of 5-AR and was therefore further examined with respect to its 5-AR binding characteristics.

5.3.1 5-AR Inhibition

With either the enzymatic assay or with the [^{3}H]4-MA binding assay, inhibition was observed within a minute after γ-LA was mixed with the microsomal enzyme preparation and was observed with both intact and detergent (polyoxyethylene ether) solubilized rat liver microsomes. As the concentration of protein increased from 2 to 20 μg, the extent of inhibition by 10 μM γ-LA decreased from 93% to 52% for intact microsomes and from 96% to 88% for solubilized microsomes.

When [^{3}H]4-MA was allowed to bind to microsomes in the presence of NADPH, followed by addition of γ-LA to a final concentration of 10 μM, about 60% of the microsome-bound [^{3}H]4-MA dissociated from the microsomes within 2 min. The remaining microsome-bound [^{3}H]4-MA dissociated at a much slower rate over the next 60 min. To determine whether γ-LA inhibition is reversible, microsomes were incubated with γ-LA and then reisolated to remove free γ-LA. The results showed that the inhibition was only partially reversed (reduced from 78% to 63% inhibition). It is possible that γ-LA was bound tightly to microsomes and/or irreversibly inactivated components which were essential for the reductase activity.

By either the enzymatic or the [^{3}H]4-MA binding assay, the inhibition could not be overcome by increasing the level of NADPH or testosterone. γ-LA did not appear to compete with testosterone or NADPH for their binding to the microsomal reductase. Double reciprocal plots of the data showed that 5 μM of γ-LA increased the apparent K_m value for NADPH (from 2.0 to 3.1 μM) and testosterone (from 2.4 to 4.5 μM), and decreased the V_{max} from 7.5 to 2.8 pmol 5α-DHT formed/mg protein/15 min. γ-LA at 5 and 10 μM increased the apparent K_i values for [^{3}H]4-MA from 13 to 20 and 40 μM, respectively, and decreased the maximal binding from 0.56 to 0.45 and 0.40 pmol/10 μg protein, respectively.

5.3.2 NADH:Menadione Reductase and UDP-Glucuronic Acid:5α-DHT Glucuronyl Transferase Inhibition

The effect of γ-LA on the activities of another microsomal reductase and a microsomal enzyme that uses a steroid as a substrate was tested to determine the specificity of the effect of γ-LA. Results showed that γ-LA at 10 to 40 μM did not affect the activities of NADH:menadione reductase or UDP-glucuronic acid:5α-DHT glucuronosyl transferase.

Mammalian 5-AR is a cellular membrane-bound enzyme. Perturbation of the lipid matrix of the membranes may affect reductase activity nonspecifically. The fact that only unsaturated fatty acids with specific configurations were potent inhibitors of 5-AR in a specific assay and that two other microsomal enzymes examined were not affected suggests that the inhibition was selective.

5.3.3 Effect of γ-LA on Human Microsomes and Prostate Cancer Cells

γ-LA inhibited NADPH-dependent [^{3}H]4-MA binding to human liver microsomes to the same degree as in experiments with rat liver microsomes. The 5α-reduction activity of [^{3}H]T by human prostate cancer cells in culture was also selectively affected by γ-LA. Table 3 shows that γ-LA, at 5 to 50 μM, inhibited 5-AR reduction of [^{3}H]testosterone in both the androgen-sensitive LNCaP cells (Horszewicz *et al.*, 1983) and the androgen insensitive PC-3 cells (Kaighn *et al.*, 1979). γ-LA, however, did not affect the metabolism of testosterone to 4-androstenedione, suggesting that 17β-steroid dehydrogenase was not sensitive to the unsaturated fatty acid. Stearic acid (5 to 20 μM) did not affect the 5-AR reduction or 17β-steroid dehydrogenase of PC-3 cells in culture.

The specific 5-AR inhibition observed with intact prostate cells in culture indicated that externally added fatty acids were able to enter cells and exert an inhibitory action on the endoplasmic reticulum or nuclear membrane-bound 5-AR *in situ*.

TABLE 3

Inhibition of the Formation of Radioactive 4-Androstenedione and 5α-DHT from [^{3}H]T by Human Prostatic Cancer Cells by γ-LA

Prostate cell line	Fatty acid added	Metabolites formed*	
		4-Androstendione	5α-DHT
	(μM)	(% of control)	(% of control)
PC-3	None	100	100
	γ-LA		
	1	102 ± 6	98 ± 6
	5	110 ± 1	50 ± 3
	20	99 ± 2	2 ± 2
	Stearic acid		
	5	103 ± 2	123 ± 2
	20	106 ± 5	121 ± 5
LNCaP	None	ND	100
	γ-LA		
	50	ND	27 ± 0
	100	ND	9 ± 4

(Table 3 continued)

*The control values for the formation of 4-androstenedione and 5α-DHT by PC-3 cells were 400,851 ± 9,507 dpm and 12,183 ± 74 dpm, respectively. The control value for the formation of 5α-DHT by LNCaP was 4,569 ± 505 dpm.

No 4-androstenedione formation was detected when LNCaP was used. γ-LA and stearic acid, at the concentrations tested, did not produce any visible change in cell morphology during the 2 hour incubation. IC_{50} values (four studies) for γ-LA with the prostate cancer cells were 10 ± 5 μM.

5.4 Example 4 -- Effects of Compounds on Androgen Action in the Hamster Flank Organ Model

The inventors sought an inhibitor of 5-AR that would be active topically and inactive systemically, as such an agent would be ideal for treatment of androgen-dependent dermatological disorders. Of the aliphatic unsaturated fatty acids tested for inhibition of 5-AR activity in liver and prostate from rats and humans, γ-LA was found to be the most potent fatty acid inhibitor when topically applied to hamster flank organs.

In this study, inhibition of androgen action by topical administration of γ-LA in hamster flank organs is investigated. Especially useful in the evaluation of the effects of these compounds on skin cells or sebaceous glands is the hamster flank organ (Frost and Gomez, 1972). The paired flank organs, one on each side of the costovertebral angle, are highly sensitive to androgen stimulation. The androgen sensitive structures in the flank organ include dermal melanocytes, sebaceous glands, and hair follicles (Hamilton and Montagna, 1950). This animal model has been widely used for testing androgenic (Hamilton and Montagna, 1950; Frost *et al.*, 1973) and antiandrogenic compounds (Voigt and Hsia, 1973; Weissmann *et al.*, 1985; Chakrabarty *et al.*, 1980). The unique advantage of this animal model is that a testing compound can be applied topically to only one of the flank organs and the effect observed on both organs. If the test compound has only a local effect then only the treated flank organ is affected. However, if the effect is systemic then both flank organs are affected. Results indicate that γ-LA applied topically inhibits androgen action locally without a systemic effect.

5.4.1 Materials and Methods

5.4.1.1 Chemicals

Fatty acids were obtained from Sigma Chemical Co., St. Louis, MO. Testosterone (T) and 5α-DHT were purchased from Steraloid, Wilton, NH.

5.4.1.2 Treatment of Castrated Animals

Pre-pubertal male Syrian golden hamsters, castrated at 4 weeks old, were obtained from Harlan Sprague-Dawley Co. (Madison, WI). Each animal was maintained individually in a plastic cage on rodent chow (Purina) and water ad libitum on a 12 h light/12 h dark cycle.

One to two weeks after castration, the hair on the lower back of each animal was clipped with an electric hair clipper and then shaved weekly to expose the flank organs. The animals were divided into 5 animals/treatment group. A treatment solution (5 μl) was applied topically to the right flank organ once a day using a Pipetteman and a polypropylene disposable tip. Unless specified the left flank organ was not treated. The treatment solution contained either (a) ethanol alone (vehicle and control), (b) an androgen (T or 5α-DHT), (c) a fatty acid, or (d) a combination of an androgen and a fatty acid. The flank organ was wiped with an alcohol pad to remove residual compound before each treatment. At the end of each experiment (17-25 days), the animals were sacrificed by either suffocation with CO_2 gas or with an intraperitoneal injection of an overdose of phenobarbital (64.8 mg/ml/animal). The flank organs, both the treated and untreated sides, were evaluated with methods to be described below to determine the effect of these treatments of the growth of the pigmented macules and the sebaceous glands. The body weight of each animal was recorded before and after treatment.

5.4.1.3 Treatment of Intact Animals

Intact male hamsters, 4 weeks old, were kept on a longer light period (16 h light/8 h dark cycle) to insure maximum stimulation of sexual characteristics (Luderschmidt *et al.*, 1984). Animals were divided into 10/group. The right flank organ was treated daily with

5 μl solution containing vehicle (ethanol) alone or γ-LA (0.5, 1, and 2 mg) for 15-25 days. The left flank organ of all animals received the same volume of vehicle.

5.4.1.4 Determination of the Area of the Pigmented Macule of the Flank Organs

The lengths of the long axis and the short axis of the pigmented spot (pigmented macule) were measured using a caliper with digital display (Digimatic, Mitutoyo Corp., Japan). The product (long axis × short axis, mm^2) was used as an index of the surface area (Wuest and Lucky, 1989). The data are presented as mean ± standard deviation.

5.4.1.5 Determination of the Sebaceous Gland Elevation

The flank organ treated with T ± fatty acid became elevated and palpable. The length of the long axis and short axis of the elevated mass were measured with a caliper. The product of the long axis × short axis (mm^2) was used as an index of the areas of the sebaceous gland, which correlated with the volume of the sebaceous glands (Weissmann *et al.*, 1984). The data are presented as mean ± standard deviation. The sebaceous glands of the flank organs, which were not treated with T, were not elevated and were not measured.

5.4.1.6 Treatment Solution

Thin layer chromatographic examinations (Whatman LK5DF silica plate using a solvent system consisting of chloroform:methanol, 3:1) of a γ-LA solution in ethanol revealed two additional more polar products, indicating oxidation of γ-LA, after 5 weeks of storage. Therefore special precautions were taken to avoid changes in treatment compounds. To avoid oxidation, all treatment compounds (T, 5α-DHT, fatty acid) were dissolved in ethanol, placed in a vial wrapped with aluminum foil to shield light, and stored at 4°C. The air in the vials were displaced with nitrogen gas by placing one or two drops of liquid nitrogen into each vial before being capped. Nitrogen was replaced each time the vials were opened. Thin layer chromatographic examination of a γ-LA solution refrigerated for 3 weeks revealed no detectable changes of the compound. All treatment solutions were prepared once a week as an additional precaution to avoid changes in the treatment solutions.

5.4.1.7 Statistics

Student's t-test was used to statistically analyze the data. A two sided p value <0.05 was considered statistically significant.

5.4.2 Results

When one of the paired pigmented macules of a prepubertal castrated male hamster was treated with either T or 5α-DHT, it became much darker in color and larger in area compared to pigmented macules treated with vehicle alone. FIG. 26 shows examples from each group of animals. Application of T or 5α-DHT to the right flank organ produced no detectable effect on the contralateral flank organ of the same animal, indicating that the effect of T and 5α-DHT stimulation is local. FIG. 27 shows one of the animals from the T treated group. T was tested at 0.5, 2, and 5 μg/flank organ/day, 5 animals/group, and the control group received vehicle alone. After 24 days of treatment, the index of the area of the pigmented macule was 2.4 ± 1.4 mm^2 for the control group, 45.6 ± 8.0 mm^2 for 0.5 μg T, 69.4 ±13.7 mm^2 for 2 μg T, and 66.4 ± 4.2 mm^2 for 5 μg T. There were no significant differences in the body weight among different treatment groups before and after treatment. A submaximal dose of T (0.5 μg/flank organ/day) was chosen for the following experiments.

γ-LA and SA were tested for their ability to inhibit the growth of the pigmented macule stimulated by T. γ-LA is the most potent fatty acid *in vitro* and SA was inactive as an inhibitor of 5-AR tested *in vitro* (Liang and Liao, 1992). The results are shown in Table 4.

TABLE 4

EFFECT OF γ-LA AND STEARIC ACID ON TESTOSTERONE-STIMULATED GROWTH OF PIGMENTED MACULE IN HAMSTER FLANK ORGAN

Treatment	Pigmented Macule (mm^2)[a]	
	Untreated (L)	Treated (R)
Control (5 μl ethanol)	5.1±1.9	4.2±0.5
γ-LA (1 mg)	4.2±0.6	4.1±0.3
Stearic Acid (SA) (1 mg)	4.4±0.4	4.9±0.9
SA (2 mg)	4.6±1.4	5.0±0.8
T (0.5 μg/5 μl ethanol)	3.6±0.5	32.7±9.2
T + γ-LA (1 mg)	4.1±0.3	15.3±3.9 (-53%, $p < 0.005$)[b]
T + SA (1 mg)	4.3±0.6	27.7±4.4 (N.S.)[+]
T + SA (2 mg)	4.2±0.4	30.1±7.1 (N.S.)c

[a]Each experimental group had 5 castrated immature hamsters. The right flank organs (R) were treated every day with 5 μl ethanol or 5 μl ethanol containing T, γ-LA, stearic acid (SA), T + γ-LA, or T + SA at the doses indicated for 18 days. The left flank organs (L) were not treated.

[b]T *vs.* T + γ-LA

[c]N.S.=not significant; T *vs.* T + SA

T treatment stimulated the growth of the pigmented macule, this effect of T was inhibited by γ-LA. This is indicated by the pigmented macules being lighter in color and smaller in area for animals treated with both γ-LA (1 mg/flank organ/day) and T (0.5 μg/flank organ/day) than those of animals treated with T alone. The pigmented area was reduced by 53% (32.7 ± 9.2 *vs.* 15.3 ± 3.9 mm^2, $p < 0.005$). In contrast, SA (1 mg and 2 mg) applied with T did not inhibit the ability of T to stimulate the growth of the pigmented macule. There were no significant differences in the pigmented macules among the control group and those treated with either γ-LA or SA alone. The body weights and contralateral flank organs were uniformly not affected. Structurally, both γ-LA and SA are aliphatic fatty acids with a chain length of 18 carbons. They are different in that γ-LA (C18:3,*cis*-6,9,12) has three *cis*-double bonds at 6, 9 and 12 positions (counting carboxyl terminal carbon as 1), and that SA (C18:0) is a saturated fatty acid without double bonds.

To further study the structural specificity of the active fatty acids, the ability of various fatty acids to inhibit T-induced growth of the pigmented macules of the flank organs was determined. Table 5 shows that γ-LA (66% inhibition) was more active than all other fatty acids tested for their ability to inhibit T-induced growth of the pigmented macule. α-LA (C18:3,*cis*-9,12,15) was less active than γ-LA (C18:3,*cis*-6,9,12), indicating that the positions of the double bonds are important for the inhibitory activity.

Oleic acid (C18:1,*cis*-9) and linoleic acid (C18:2,*cis*-9,12) were active, whereas their transisomers, elaidic acid (C18:1,*trans*-9) and linolelaidic acid (C18:2,*trans*-9,12) were inactive suggesting that fatty acids with a *cis* double bond configuration are more active than those with a *trans* configuration. Weak inhibitions were found with palmitic acid (C16:0), arachidonic acid (C20:4,*cis*-5,8,11,14), and erucic acid (C22:1,*cis*-13). There was no significant inhibition by undecylenic acid (C11:1,10) or nervonic acid (C24:1,*cis*-15). Fatty acid specificities *in vivo* are, in general, similar to their ability to inhibit the 5-AR activity *in vitro* (Liang and Liao, 1992). In the absence of T, none of the fatty acids tested stimulated or inhibited the growth of the pigmented macules.

To investigate whether inhibition of 5-AR is the primary mode of action of γ-LA, the ability of γ-LA to inhibit T and 5α-DHT-induced growth of the hamster flank organ was compared γ-LA was tested at dosages from 0.01 to 2 mg/flank organ/day for their

ability to inhibit the growth of the pigmented macule stimulated by T (0.5 μg/flank organ/day). γ-LA was found to be effective at a dosage of 0.2 mg or higher and a maximal inhibition (50%) was reached by 1 mg. γ-LA (0.2 to 1 mg/flank organ/day) was tested for its ability to inhibit T and DHT induced growth of the pigmented macule and the results are shown in Table 6. These results indicate that T-induced growth of the pigmented macule was preferentially inhibited by γ-LA. There is no statistically significant inhibition of 5α-DHT-induced growth by γ-LA although the average values were reduced by higher dosages of γ-LA.

TABLE 5

EFFECTS OF FATTY ACIDS ON
TESTOSTERONE-STIMULATED GROWTH OF PIGMENTED MACULE

Fatty Acid	Pigmented macule[a] Left (mm^2)	Pigmented macule[a] Right (+T) (mm^2)	Percent Inhibition	p
None	4.8±1.1	49.6±8.3	–	–
Undecylenic acid (C11:1,10)	4.7±1.2	41.7±10.7	–	N.S.[b]
Palmitic acid (C16:0)	5.1±2.9	37.2±6.3	25%	<0.05
Oleic acid (C18:1,*cis*-9)	4.7±0.5	28.1±8.3	43%	<0.005
Elaidic acid (C18:1,*trans*-9)	4.9±1.1	47.0±5.9	–	N.S.
Linolenic acid (C18:2,*cis*-9-12)	5.4±1.6	23.9±5.0	52%	<0.001
Linolelaidic acid (C18:2,*trans*-9,12)	4.1±0.7	46.6±8.7	–	N.S.
α-LA (C18:3,*cis*-9,12,15)	4.1±1.2	27.6±8.2	44%	<0.005
γ-LA (C18:3,*cis*-6,9,12)	4.4±1.6	17.0±6.4	66%	<0.001
Arachidonic acid (C20:4,*cis*-5,8,11,14)	4.7±1.6	35.7±7.9	28%	<0.05
Erucic acid (C22:1,*cis*-13)	4.6±1.5	35.4±4.6	29%	<0.02
Nervonic acid (C24:1,*cis*-15)	4.0±1.5	39.9±5.4	–	N.S.

(Table 5 continued)

[a]The right flank organ was treated topically with 5 μl solution containing either testosterone (0.5 μg) or testosterone plus a fatty acid (1 mg). The left flank organ received same solution as the right flank organ except that testosterone was omitted. The treatments were once a day for 21 days. Five animals per treatment group.

[b]N.S. = not significant

TABLE 6

DIFFERENTIAL EFFECT OF γ-LA ON TESTOSTERONE- AND 5α-DHT-INDUCED GROWTH OF PIGMENTED MACULES AND SEBACEOUS GLANDS OF FLANK ORGANS

Treatment	Pigment Macule (mm^2)	P	Sebaceous Gland (mm^2)	P
$T_{0.5\mu g}$	32.9±2.6	-	45.8±4.1	
$T + \gamma\text{-}LA_{0.2mg}$	22.7±4.3 (-31%)	<0.005	32.2±6.0 (-30%)	<0.005
$T + \gamma\text{-}LA_{0.4mg}$	24.9±5.8 (-24%)	<0.02	32.2±17.2	N.S.[b]
$T + \gamma\text{-}LA_{0.6mg}$	18.0±3.4 (-45%)	<0.001	22.6±3.0 (-51%)	<0.001
$T + \gamma\text{-}LA_{0.8mg}$	22.3±2.3 (-32%)	<0.001	34.5±8.4 (-25%)	<0.005
$T + \gamma\text{-}LA_{1.0mg}$	21.6±2.3 (-34%)	<0.001	20.4±3.0 (-55%)	<0.001
$DHT_{0.5\mu g}$	33.7±8.6	-	30.3±4.5	-
$DHT + \gamma\text{-}LA_{0.2mg}$	32.1±3.1	N.S.	39.5±6.9	N.S.
$DHT + \gamma\text{-}LA_{0.4mg}$	28.9±3.2	N.S.	35.8±13.1	N.S.
$DHT + \gamma\text{-}LA_{0.6mg}$	27.0±3.9	N.S.	39.7±10.7	N.S.
$DHT + \gamma\text{-}LA_{0.8mg}$	24.9±5.3	N.S.	30.3±12.9	N.S.
$DHT + \gamma\text{-}LA_{1.0mg}$	24.7±3.0	N.S.	33.3±11.9	N.S.

[a]The right flank organs of castrated immature male hamsters were treated topically with a 5 μl solution ethanol containing T or 5α-DHT alone or a combination of T and γ-LA or 5α-DHT and γ-LA. The treatment was once a day for 19 days. The data

(Table 6 continued)

shown are for the right (treated) flank organ. The left organ was not treated. The amount of androgens and γ-LA used each day are indicated.

[b]N.S. = not significant

Table 6 also shows differential effects of γ-LA on the T- and 5α-DHT-induced growth of the sebaceous glands, recognizable as palpable mass, in the flank organs. The sebaceous glands are directly underneath the pigmented macule, but extend beyond the pigmented area after T treatment. γ-LA also inhibited T-induced growth of the sebaceous glands, but it did not significantly affect the growth of the sebaceous glands stimulated by 5α-DHT treatment.

It was determined whether γ-LA could inhibit the growth of the pigmented macule stimulated by endogenous androgens as intact male hamsters developed from a sexually immature to mature status. The right flank organs were treated daily with vehicle alone or γ-LA (0.5 and 1 mg). The left flank organs of animals in all three groups received vehicle alone. At the beginning of treatment, the pigmented macules of all groups were small, approximately 4 mm^2. After 15 days of stimulation by endogenous androgens, the pigmented macules of the control group grew 6-fold, with no difference between the right and left side. γ-LA treatment significantly inhibited the growth of the pigmented macule (Table 7). FIG. 13 shows one of these specimens. The inhibition by γ-LA treatment was local since the growth of the contralateral pigmented macules was not affected (FIG. 14 and Table 4). γ-LA treatment also inhibited hair growth of the treated flank organ.

FIG. 15 shows the representative examples which indicate that the hair in the γ-LA treated flank organ was lighter in color and shorter in length than those of the control. In another study, the right flank organs of intact immature male hamsters received daily treatment with vehicle alone (control), 1 mg γ-LA, and 2 mg γ-LA. The areas of the pigmented macules were calculated from periodic measurement to demonstrate the inhibition of the growth rate of the pigmented macule by γ-LA treatment. FIG. 16 shows that the pigmented macules of the control animals grew linearly until day 16, but this growth was dramatically reduced by γ-LA treatment. The growth rates of the pigmented macules of the left flank organs were similar among all three groups, indicating that the inhibitory effect of γ-LA treatment was local.

TABLE 7

INHIBITION OF GROWTH OF THE PIGMENTED MACULE IN INTACT MALE HAMSTERS BY γ-LA

Animal	Left flank organ		Right flank organ	
	Treatment	Pigmented macule (mm^2)	Treatment	Pigmented macule (mm^2)
Group 1	vehicle	24.7±2.1	vehicle	23.5±3.8 (N.S.)[#]
Group 2	vehicle	28.6±4.0 (N.S.)[b]	γ-$LA_{0.5mg}$	19.0±3.6 (-34%, $p<0.001$)[#] (-19%, $p<0.0252$)[*]
Group 3	vehicle	29.2±4.8 (N.S.)	γ-$LA_{1.0mg}$	10.8±2.7 (-63%, $p<0.001$)[#] (-54%, $p<0.001$)[*]

[a]The left flank organ of all groups received ethanol only. There were 10 animals/group. The results of the γ-LA treated groups were compared either with that of group 1 or with the left flank organ of the same group.

[b]N.S. = not significant

[c]The right flank organs of intact male hamsters at 4 weeks of age were treated with a topical solution containing either 5 μl ethanol or 5 μl ethanol containing 0.5 or 1.0 mg γ-LA every day for 15 days.

[d]T *vs.* T+γ-LA

5.4.3 Discussion

Topical applications of certain unsaturated fatty acids such as γ-LA can inhibit androgen action in the hamster flank organ. Several lines of evidence suggest that inhibition of 5-AR may be the primary mode of action for γ-LA. First, in the cell-free systems, γ-LA is a potent inhibitor of 5-AR in liver or prostate from humans and rats (Liang and Liao, 1992). Second, in castrated hamsters γ-LA inhibits the growth of the pigmented macule induced by T, but not that induced by 5α-DHT. Third, the structural requirements for fatty acids to inhibit T-induced growth of the pigmented macules and to inhibit 5-AR are very similar. In addition to its ability to inhibit T-induced growth of the pigmented macule, γ-LA also inhibits T-induced growth of other structures of the flank organ *e.g.*, the sebaceous glands and hair. This suggests that γ-LA acts on 5-AR, which is a common step in androgen action in all three structures. This demonstrates specific effect of unsaturated fatty acids on androgen action *in vivo*.

Topically applied γ-LA did not completely abolish the growth of the pigmented macule in castrated hamsters treated topically with T. The maximum inhibition (50 to 66%) was reached with the daily dose of 1 to 2 mg γ-LA. Several factors may contribute to this incomplete inhibition. First, the penetration of γ-LA may be a limiting factor. Second, T can affect androgen action by binding to androgen receptors, although with lower receptor binding affinity than that of 5α-DHT (Liao *et al.*, 1973).

γ-LA, an essential fatty acid (Horrobin, 1992), and the active unsaturated fatty acids shown in this study are normal components in human tissue, *e.g.*, skin (Schafer and Kragballe, 1991). Therefore, they should be safe to use in humans. Since γ-LA applied topically produced a localized effect without systemic action, γ-LA and its analogues are desirable for topical application and treatment of androgen-dependent skin conditions such as acne, androgenetic alopecia, female hirsutism, sebaceous hyperplasia, and seborrhea.

5.5 Example 5 -- Effects of γ-LA Injection to Hamsters on Organ Growth

5.5.1 γ-LA Inhibits Growth of Flank Organs

Before treatments, the areas of pigmented spots of flank organs were similar between the two groups. The right flank organ was 5.9 ± 1.2 mm^2 for the control group and was 6.6 ± 2.0 for the γ-LA group. The left flank organ was 6.2 ± 1.4 for the control group and was 6.2 ± 2.1 for the γ-LA group. γ-LA injections inhibited the growth of the flank organ, both the right and the left.

Thus, the right flank organ was 14.6 ± 1.5 mm^2 for the control group and was 9.8 ± 2.5 for the γ-LA group ($p < 0.001$). The left flank organ was 12.9 ± 2.2 for the control group and was 9.9 ± 2.4 for the γ-LA group ($p < 0.02$).

5.5.2 γ-LA Inhibits Growth of Seminal Vesicles and Prostate

These two tissues were weighed together because the prostates were hard to separate from the seminal vesicles. The seminal vesicles and prostates were 0.156 ± 0.026 g for the control group and were 0.106 ± 0.022 for the γ-LA group ($p < 0.001$).

5.5.3 γ-LA Does Not Inhibit Growth of Kidneys, Adrenal or Spleen

The weights for each of these organs were: kidney (1.060 ± 0.086 g for the control group *vs.* 1.121 ± 0.073 for γ-LA group), adrenal (0.022 ± 0.004 g for control *vs.* 0.021 ± 0.004 for γ-LA), or spleen (0.157 ± 0.027 for control *vs.* 0.1867 ± 0.048 for γ-LA). None of the differences between the control and γ-LA groups are statistically significant.

γ-LA given to hamsters by subcutaneous injections inhibited the growth of androgen-dependent tissues: the flank organs, seminal vesicles and prostates. γ-LA treatments did not affect the kidney, adrenal, or spleen. These tissues are known not requiring androgen to grow. Thus, γ-LA administered subcutaneously can inhibit androgen actions.

5.6 Example 6 -- Topical Effects of Compounds on Hair Loss and Growth

The stamptail macaque monkey develops baldness in a pattern resembling human androgenetic alopecia. The balding process begins shortly after puberty (approximately 4 years of age). This occurs in nearly 100% of the animals, males and females, and is androgen dependent. This is a useful animal model for human androgenetic alopecia and is contemplated to be useful in demonstrating the effects of polyunsaturated fatty acids on hair loss. The following describes a protocol for testing (Rittmaster *et al.*, 1987; Diani *et al.* 1992)

Male stamptailed macaques (4 years of age) are divided into groups of 3 to 5 animals. A defined area of the scalp involving the frontal and vertex areas is marked, *e.g.*, by tattoo. Hairs in the marked area are shaved. The solutions of a testing compound in different dosages and combinations are evenly applied to the shaved areas once or twice a day. Control animals receive the same volume of the solvent (*e.g.*, ethanol or other organic solvent, or a cream). The same area of the scalp is shaved every 4 to 6 weeks and the weights of hairs shaved are determined. The treatments may last for 6 months to 2 years. 4-MA (17-N,N-diethylcarbamoyl-4-methyl-4-aza- 5-androstan-3-one), a 5-AR inhibitor known to prevent baldness in this animal is included as a positive control. Biopsies of the scalp (4-mm punch) are obtained before and at the end of the treatments. The specimens are analyzed for 5-AR activity and examined histologically for evidence of alopecia.

5.7 Example 7 -- Effects of Catechins on 5-AR Activity

By enzymatic assay, tea catechin gallates are potent inhibitors of the type 1 but not type 2 5-AR. The IC_{50} for (-)Epigallocatechin-3-gallate and (-)epicatechin-3-gallate for the type 1 human 5-AR are about 10 μM. (-)Epicatechin and (-)epigallocatechin are not active for either types of the enzyme. Unlike 4-azasteroids and many other nonsteroidal inhibitors, these gallates do not contain heterocyclic nitrogen rings and do not compete with testosterone or NADPH for binding to 5-AR.

5.7.1 Experimental Procedures

5.7.1.1 Materials

Various biochemicals and polyphenolic compounds were obtained from Sigma Chemical Co. (4-^{14}C)-T (60 mCi/mmol) was a products of New England Nuclear. (1,2-^{3}H)17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one ([^{3}H]4-MA) (60 Ci/mmol) was prepared as previously described (Liang *et al.*, 1983). Purified catechins were obtained from Funakoshi Co. (Tokyo, Japan) or from Sigma Chemical Co. The purity of individual catechins was at least 98% pure based on the NMR and HPLC analyses. Various catechins were also purified in the inventor's laboratory from green tea (*Camellia sinensis*) as described below.

5.7.1.2 Isolation and Purification of Catechins from Green Tea

Dried green tea (50 g) was extracted twice with 500 ml of 90°C H_2O for 15 ~30 min. The combined water extract was freeze dried. The dried powder was dissolved in 50 ml of water and extracted with an equal volume of $CHCl_3$. catechins in the aqueous layer were extracted twice with ethyl acetate. After removal of ethyl acetate, the dried powder (1 g) was dissolved in 10 ml of 95% ethanol and loaded onto a Sephadex LH-20 column (5 × 35 cm). The column was eluted with 95% ethanol and the effluent was monitored by UV absorption at 280 nm. The identity and purity of isolated catechins (FIG. 21) were assessed by NMR spectrum analyses and HPLC. HPLC was performed by using a C18 reversed phase column (4.6 mm × 250 mm, 5 μ, Alltech Co.) isocratically with acetonitrile/ethyl acetate/0.05% H_3PO_4 in water (12:2:86) as the mobile phase.

5.7.1.3 Preparation of Rat 1A Cells Expressing Human 5-ARs

The cDNAs for the human type 1 and 2 5-ARs were isolated from human prostate λZAP II™ cDNA libraries using the published sequence of the 5-ARs, PCR™ and standard library screening techniques (Sambrook *et al.*, 1989). The type 1 and 2 cDNAs correspond to nucleotides 31-870 and 28-829, respectively, of the published sequences for these 5-ARs (Anderson and Russell, 1990; Anderson *et al.*, 1991). The type 1 and 2 cDNAs were subcloned into the retroviral expression vector pMV7 (Kirschmeier *et al.*, 1988) and high

titer stocks of virus containing the type 1 and 2 cDNAs were generated using the packaging cells BOSC 23 293 (Kirschmeier *et al.*, 1988). Rat-1A cells (Pear *et al.*, 1993) were infected with virus (Topp, 1981) and cells containing integrated retrovirus were selected for G418-SO_4 resistance, (Brown and Scott, 1987).

5

5.7.1.4 [^{3}H]4-MA Binding and Enzymatic Assays for Microsomal 5-AR

Microsomes were prepared as described previously (Liang and Liao, 1992) from the liver of adult Sprague-Dawley female rats or Rat-1A cells expressing a specific type of human 5-AR. Each study was carried out in duplicate or triplicate which were usually within 10% of each other. Several studies were preformed to assure that the results shown are representative. The data presented was based on the rate of reaction. The compounds showing less than 10% inhibition were considered not active (NA) at the indicated concentrations.

[^{3}H]4-MA binding assay were described in detail previously (Liang and Liao, 1992; Liang *et al.*, 1983). The assay solution, in a final volume of 0.15 ml, contained 0.08 μCi of [^{3}H]4-MA, 0.1 mM NADPH, 0.1 mM dithiothritol and 50 mM potassium phosphate, pH 7.0, with or without a test compound. The reaction was started by the addition of 25 μl of microsomes (25 μg protein). After 30-60 min incubation at 0°C, the [^{3}H]4-MA bound to microsomes was collected on a Whatman GF/F glass fiber, washed and radioactivity determined (Liang and Liao, 1992).

The enzymatic assay was based on the measurement of 5α-DHT production from testosterone in the presence of microsomes (Liang and Liao, 1992; Liang *et al.*, 1983). The assay mixture, in the final volume of 0.25 ml, contained 2.8 μM 4-[^{14}C] testosterone, 0.1 mM NADPH, 1 mM dithiothreitol, and 100 mM potassium phosphate, pH 6.0, with or without a test compound. The reaction was started by the addition of 25 μl of microsomes (25 μg protein). The mixture was incubated at 37° for 30-60 min and stopped by addition of 0.5 ml of ethyl acetate and mixing for 1 min. The organic solvent extract was removed under vacuum. The dried extract was dissolved in 25 μl of ethyl acetate and applied to a silica gel 60 TLC plate which was developed in a solvent system consisting of methylene chloride:ethyl acetate:methanol (85:15:3). Conversion of testosterone to 5α-reduced

metabolites was measured by scanning the TLC plate on an AMBIS radioanalytical scanner. 5α-DHT was the predominant metabolite (>95%) with little or no conversion of testosterone to androstanediols, androstandione, 4-androstenedione or thermetabolites.

5.7.2 Results

Most of the 5-AR activity is lost during the solubilization and purification of 5-AR from microsomal or nuclear membranes. The 5-AR activity, therefore, has been characterized by measuring the rate of conversion of testosterone to 5α-DHT by whole cells or by microsomal preparations in the presence of NADPH. The 5-AR activity of rat liver which contains only the type 1 5-AR (Russell and Wilson, 1994; Normington and Russell, 1992) can also be reliably assayed by following NADPH-dependent noncovalent binding of a potent radioactive inhibitor, such as [^{3}H]4-MA, which strongly competes with testosterone for binding to the reductase (Liang *et al.*, 1983).

Based on the [^{3}H]4-MA binding assay (Table 8) or enzymatic assay EGCG, ECG and CG but not (+)catechin, (±)catechin, (-)gallocatechin, (-)epicatechin, or (-)epigallocatechin are potent inhibitors of the type 1 5-AR of rat liver microsomes. The IC_{50} for EGCG, ECG, and CG were 3 μM, 12 μM and 18 μM, respectively. Since the presence of the gallate moiety in the catechin molecule appeared to be important for the inhibitory activity, a number of compounds having the gallate structure were tested. Gallic acid and a number of alkyl gallates were not active even at 200 μM. Several compounds having a gallolyl (3,4,5-trihydroxy benzene) group were also not inhibitory (Table 8).

TABLE 8

INHIBITION OF [^{3}H]4-MA BINDING TO 5-AR OF RAT LIVER MICROSOMES BY TEA CATECHINS AND RELATED COMPOUNDS

Test Compound	% Inhibition of [^{3}H]4-MA binding				
	Concentration of test compound (μM)				
	5	10	20	40	200
(+)Catechin	NA[a]	NA	NA	NA	NA
(±)Catechin	NA	NA	NA	NA	NA
(-)Gallocatechin	NA	NA	NA	NA	NA
(-)Epicatechin	NA	NA	NA	NA	NA
(-)Epigallocatechin	NA	NA	NA	NA	NA
(-)Catechin-3-gallate	12	31	53	73	91
(-)Epicatechin-3-gallate	25	44	74	88	92
(-)Epigallocatechin-3-gallate	61	74	79	88	93

[a]No inhibitory activity was found with the above compounds having gallolyl (3,4,5-trihydroxybenzene) or gallate groups at concentrations up to 200 μM: pyrogallol, 3,4,5-trimethoxy benzoic acid, 3,4,5-trimethoxy phenylacetic acid, 3,4,5-trihydroxy-benzamide, 3-[3,4,5-trimethoxyphenyl] propionic acid, gallic acid, methyl gallate, *n*-propyl gallate, isopropyl gallate, *n*-octylgallate, and *n*-dodecyl gallate. Among other compounds having a similar structure but are inactive are: quercetin, α-naphthaflavone, β-naphthaflavone, rutin, 4[4-chlorophenylmethyl]-6,7-dimethoxy-isoquinoline methanesulfonate. Caffeine, a major compound in the tea extract was also not active at concentrations up to 200 μM.

[b]NA = not active.

By the [^{3}H]4-MA assay (Table 9) or the enzymic assay (Table 10), (-)epicatechin, and (-)epigallocatechin were not active inhibitors for either type 1 or 2 human microsomal 5-AR that were genetically engineered and expressed in Rat-1A cells. By the [^{3}H]4-MA binding assay (Table 9), ECG and EGCG, were active inhibitors of both isozymes but exhibited some preferential inhibition of the type 1 isozyme at concentrations below 30 μM. However, by the enzymic assay (Table 10), ECG and EGCG, at concentrations below 30 μM, did not inhibit the Type 2 isozyme but were potent inhibitors for the Type 1 isozyme. The IC_{50} values for the two gallates were about 10 μM. As expected, finasteride which has been shown to selectively inhibit type 2 human isozyme (2) inhibited the type 2 isozyme expressed in Rat-1A cells. In comparison, γ-LA inhibited both types of human isozymes, either based on the [^{3}H]4-MA binding assay (Table 9) or by the enzymic assay (Table 10).

TABLE 9

INHIBITION OF [^{3}H]4-MA BINDING TO HUMAN 5-AR ISOZYMES BY VARIOUS COMPOUNDS

Test compound	Isozyme type	% Inhibition of [^{3}H]4-MA binding				
		Concentration of test compound (μM)				
		1	3	10	30	100
(-)Epicatechin	1	NA	13	NA	NA	18
(-)Epicatechin	2	NA	NA	NA	NA	NA
(-)Epigallocatechin	1	NA	NA	NA	NA	23
(-)Epigallocatechin	2	NA	NA	NA	13	14
(-)Epicatechin-3-gallate	1	34	47	79	87	79
(-)Epicatechin-3-gallate	2	17	17	45	76	80
(-)Epigallocatechin-3-gallate	1	29	47	77	88	86
(-)Epigallocatechin-3-gallate	2	NA	12	55	72	89
γ-LA	1	20	54	65	79	85
γ-LA	2	22	49	63	72	88
Finasteride[a]	1	NA	NA	NA	NA	NA
Finasteride[a]	2	NA	NA	22	44	67

[a]Concentration in nM

With the enzymatic assay or the [^{3}H]4-MA binding assay, ECG and EGCG inhibited the rate and extent of the reaction by type 1 isozyme of the rat liver or Rat-1 cells. These gallates did not appear to compete with testosterone or NADPH for their binding to microsomal 5-AR since the inhibition could not be overcome by increasing the concentration of NADPH or testosterone. These gallates did not induce NADPH oxidation during the incubation of the reaction mixtures in the presence of microsomes.

Most of the known 5-AR inhibitors are steroid derivatives (Russell and Wilson, 1994; Faller *et al.*, 1993). Finasteride, is a therapeutic agent for treatment of benign prostate hyperplasia (BPH) (McConnel *et al.*, 1992). 4-MA and finasteride are 4-azasteroids that are effective in preventing male pattern baldness in experimental animals (Rittmaster *et al.*, 1987; Rittmaster, 1994). LY191704, a selective inhibitor of the type 1 5-AR, shares the structural feature of 4-azasteroids but lacks the fourth ring of a steroid (Hirsch *et al.*, 1994). ONO-3805 (Russell and Wilson, 1994) is one of several benzoylaminophenoxybutanoic acid derivatives that posses the 5-AR inhibitory activity. 4-azasteroids, LY191704, and ONO-3805 contain amino groups that may play a role in their interaction with 5-AR. In contrast, unsaturated fatty acids may act by perturbation of the lipid matrix of the membranes and, therefore, are not selective inhibitors of 5-AR isozymes (Russell and Wilson, 1994). The isozyme-dependent effect of tea gallates suggests that the galloyl group can interact with a specific group in the type 1 5-AR. Since many alkyl gallates were not active (Table 8), other structural features in the epicatechin molecules may be also important for inhibition.

It has been was shown that γ-LA can inhibit the conversion of testosterone to 5α-DHT by human prostate cancer (PC3 and LNCaP) cells in culture; EGCG also reduced 5α-DHT production by these cells in culture. EGCG administered to male rats also was able to reduce the weight of ventral and dorsolateral prostates, coagulating glands, seminal vesicles, and preputial glands without affecting the weight of testis or kidney.

Some catechins have been shown to inhibit enzymatic activity *in vitro*. The most sensitive enzyme reported was HIV-1 reverse transcriptase (IC_{50} for EGCG = 40 nM) (Nakane *et al.*, 1994). However, this inhibition is apparently non-specific (Moore and Pizza, 1992). Soybean lipoxygenase is also inhibited by EGCG (IC_{50} = 10 μM), ECG (IC_{50} = 18

μM), and EGC (IC_{50} = 21 μM). α-Amylase of human saliva, rat small intestinal sucrase and maltase are less sensitive to EGCG inhibition (IC_{50} = 50-500 μM) (Honda *et al.*, 1994).

Various tea catechin gallates and related compounds have anticarcinogenic activity against cancer of the esophagus, skin, colon and other organs (Yang and Wang, 1993). ECG and EGC account for about 65% of solid matter in the hot water extract of green tea. Their concentrations in green tea beverage are about 5-10 mM.

TABLE 10

INHIBITION OF 4-[^{14}C]TESTOSTERONE REDUCTION BY HUMAN 5-AR ISOZYMES BY VARIOUS COMPOUNDS

Test compound	Isozyme type	% Inhibition of 5-AR				
		Concentration of test compound (μM)				
		1	3	10	30	100
(-)Epicatechin	1	NA	13	NA	NA	14
(-)Epicatechin	2	NA	NA	NA	NA	NA
(-)Epigallocatechin	1	NA	NA	NA	NA	15
(-)Epigallocatechin	2	NA	NA	NA	NA	NA
(-)Epicatechin-3-gallate	1	20	27	47	90	99
(-)Epicatechin-3-gallate	2	NA	NA	NA	NA	82
(-)Epigallocatechin-3-gallate	1	22	23	34	89	98
(-)Epigallocatechin-3-gallate	2	NA	NA	NA	NA	73
γ-LA	1	31	52	89	99	99
γ-LA	2	30	48	60	88	99

5.8 Example 8 -- Effects of Compositions on Sebum Production in a Human Model

Topical antiandrogenic activity of several fatty acid and catechin was first evaluated in the hamster flank organ assay or the rat assay. To further confirm the effectiveness of antiandrogenic compounds and suitability for human use, tests were performed on a human male subject. The ideal compounds for human treatment are those that are topically and locally active but do not show systemic antiandrogenic activity, especially in the cases involving young males. In the following example, two classes of compounds were tested by measuring sebum secretion from the forehead of an adult male treated topically.

5.8.1 Methods

5.8.1.1 Determination of Forehead Sebum Production

A 63-year old Asian male volunteer was used to test and analyze sebum production from the forehead region. The forehead was washed thoroughly by soap twice and cleaned by 70% isopropyl alcohol twice. Sebum production was measured 30 to 60 minutes later by a sebum meter (Courage/Khazaka Electronic GmbH, Germany). The sebum meter tape probe (7 mm × 8 mm) covered 56 mm^2 area in each measurement. Ten measurements were made within the 4 cm square area (16 cm^2) located at the middle of the left or right side forehead between the eyebrow and the hair line.

The sebum meter detected the difference in the transparency of the tape before and after the tape was placed on the forehead for 30 seconds and expressed the difference in an arbitrary number (S-value) between 0 to 300 (or higher). S-values of sebum accumulated on the foreheads of men are usually 200 to 300. Skin surface on hands usually showed a very low number (5 to 20). The S-value for forehead immediately after washing was less than 5. For men, the S-value gradually increased to about 50 within 30 minutes after washing and reached 100 to 200 in 45 minutes to 55 minutes.

To determine the rate of sebum production, the left and the right forehead areas were measured alternatively and each time at the comparable areas on the two sides. Ten measurements on each side (*i.e.*, 20 measurements for two sides) could take about 15-20 minutes and the sebum-values ranged between 30 to 200. The S-values were different

considerably at different areas of the forehead and could be influenced by environmental, including weather, diet, and physiological conditions. However, the ratio of the total S-value (the sum of 10 measurements) for the left and the total S-value for the right forehead was constant. For the Asian male tested in this experiment, the L/R ratios measured over a six month's period was within 1.15 to 1.38 if the S-values were determined 30 to 50 minutes after the forehead was washed thoroughly. Therefore, compounds applied to the left forehead that reduced the L/R ratio to lower than 1.1 were considered as topically active agents for suppression of sebum production.

5.8.2 Results

5.8.2.1 γ-LA Inhibition of Human Forehead Sebum

In the experiment shown in FIG. 8, 0.2 ml of borage oil (containing 18% of γ-LA) in a gel capsule was applied to the left forehead twice daily for 23 days. During this period, L/R ratio reduced from 1.28 ± 0.03 down to 1.05 ± 0.01. After the borage oil treatment was stopped, the L/R ratio returned to 1.20 ± 0.14. The effect of borage oil on the sebum production on the left forehead was relatively small, possibly due to the fact that most γ-LA in the borage oil was in the form of triglyceride that did not inhibit 5-AR. Free acid released from the glyceride by nonenzymic or enzymic action was probably responsible for the effect.

After the borage oil application was stopped and the L/R ratio recovered to 1.33, 20 mg of pure γ-LA was applied to the left forehead twice each day for 6 days. The L/R ratio decreased to 0.22 during this period. After the γ-LA application was stopped, the L/R ratio recovered slowly to 1.20 ± 0.14 over the period of 16 days. The finding clearly showed that γ-LA was superior than borage oil in quickly suppressing sebum production from forehead of a human male subject.

5.8.2.2 Catechin Inhibition of Human Forehead Sebum Production

Twenty mg of (−)epigallocatechin gallate (EGCG) in 0.2 ml 70% ethanol was applied to the left forehead twice a day for 6 days (FIG. 9). The L/R ratio decrease from 1.20 ± 0.02 to 0.71 ± 0.04 during this period. After the EGCG treatment was stopped, the L/R ratio gradually recovered to 1.19 ± 0.02 within 16 days. Subsequent treatment of the left forehead with 20 mg (−)epicatechin in 0.2 ml 70% ethanol twice a day for 6 days reduced the L/R ratio to 1.02 ± 0.1. After the application was stopped, the L/R ratio gradually increased to the normal value of 1.21 ± 0.01 in 10 days. Clearly EGCG was more effective in reducing the sebum production from forehead than (−)epicatechin.

5.9 Example 9 −− Effects of Catechins on Body Weight and Androgen−Dependent Organs in the Rat

Male Sprague−Dawley rats (body weight 180 g ± 10 g) were used. Each group had 5 rats. EGCG (15 mg/0.1 ml 30% ethanol/rat/day) was intraperitoneally injected into rats in one group each day for 7 days. Rats in the control group received 0.1 ml 30% ethanol. The results of this study is presented in Table 11.

5.9.1 Effects on Body Weight, Prostate Growth, and the Preputial Organ in Rats

Male Sprague−Dawley rats, 60 ± 5 g body weight, were used. Each group had 6 rats, and all were castrated. On the day of castration, and thereafter, (−)epicatechin (EC), (−)epigallocatechin gallate (EGCG), or γ−LA (5 mg each in 0.1 ml of 30% ethanol containing 10 μg testosterone) was injected intraperitoneally every day for 14 days. The control rats received 0.1 ml of 30% ethanol containing 10 μg testosterone daily.

TABLE 11

EFFECT OF EPIGALLOCATECHIN GALLATE ON BODY WEIGHT AND MALE ACCESSORY REPRODUCTIVE ORGANS, AND PREPUTIAL ORGANS OF RATS

	Control	EGCG[a]	% of Control	
			Final[b]	Increase[c]
Final body weight (g)	238.2 ± 10.6	191.8 ± 12.0	80.5	20.3
Ventral prostate (mg)	201.9 ± 36.2	142.2 ± 10.7	70.4	62.0
Seminal Vesicle (mg)	234.3 ± 11.7	164.9 ± 30.4	70.4	52.3
Coagulating gland (mg)	78.0 ± 5.9	45.5 ± 11.7	58.3	39.8
Preputial gland (mg)	126.8 ± 24.8	76.1 ± 33.1	60.0	−28.2[d]

[a]15 mg/day.
[b]Based on the final weight.
[c]Based on the increase in the weight.
[d]Reduced from the control weight.

The results showed that γ-LA and EGCG but not EC reduced the weights of ventral prostate and preputial organ by about 10 to 35%. EGCG but not EC or γ-LA reduced the body weight increase by about 35%. It appears that γ-LA and EGCG will be useful in reducing the prostate size where as EGCG may be useful in the control of body weight increase. Rats injected with EGCG or γ-LA appeared to be as healthy as rats in other groups. When various organs were examined by eyes, there was no obvious infection, necrosis or changes in the color or the size of organs such as adrenal, spleen, liver, kidney, thymus, pancreas, *etc.* Fat content under the skin appeared less in the rats injected with EGCG. EGCG effect on the prostate weight or body weight increase may be specific, at least in part, due to the modulation of androgen-dependent growth of prostate, muscle growth or cellular fat production.

5.9.2 Catechins Affect Body Weight, Growth of Prostate and Preputial Organs in Large Rats

Male Sprague Dawley rats, 172 ± 20 g body weight, were used. Each group had 5 rats. EC, EGC, ECG, or EGCG, (15 mg each in 0.3 ml of 30% ethanol) was injected intraperitoneally every day for 7 days. The control rats received 0.3 ml of 30% ethanol.

The results showed that while the normal rats gained body weight, during the 7 days of experiment, from 172 ± 20 g to 232 ± 10 g (about 35% increase), the body weight of rats receiving EGCG decreased to an average of 147 ± 14 g (about 15% decrease). Thus, rats in the EGCG group were about 36% smaller than the normal rats at the end of the study. The effect of ECG and other catechins on the body weights was less than 10%. At the end of the study, the organ weights of rats in the EGCG group in compared to those of normal rats were significantly decreased: (% of organ weight in normal rats): preputial gland (35%), ventral prostate (46%), dorsolateral prostate (46%), seminal vesicle (41%), coagulating gland (34%), testis (84%), and kidney (74%). The results indicated that EGCG is effective in reducing lipid or sebum producing organ such as preputial gland and male hormone-sensitive organs, such as ventral and dorsolateral prostate gland, coagulating gland and seminal vesicles. Since ECG that are structurally very similar to EGCG (ECG has one -OH group less than EGCG) are considerably less effective than EGCG, the EGCG effect on

lipid production or organ weights may be dependent on a highly specific EGCG interaction with a macromolecule(s) that can be considered a specific receptor for EGCG or a protein complex which regulate enzyme activities, gene expression and organ growth. Regulation or modulation, by natural or synthetic compounds, of the interaction or the function of the EGCG-receptor (or protein) complex may be utilized to control the lipid synthesis or the growth and function of androgen-dependent organs such as prostates.

5.9.3 Effect of EGCG on Body Weight and Androgen-Dependent Growth of Prostate and Preputial Glands of Rats

Male Sprague-Dawley rats, 60 ± 5 g body weight, were used. Rats were divided into 6 groups. Each group had 5 rats. Groups 1 and 2 were normal rats while the other 4 groups had rats castrated on the first day. On the first day and every day thereafter, Groups 3 and 4 received 100 μg testosterone/day and Groups 5 and 6 received 100 μg 5α-DHT/day. In addition Groups 2, 4, and 6 received 5 mg EGCG each day. Androgens and/or EGCG were dissolved in 0.1 ml of 30% ethanol and injected intraperitoneally daily for 7 days. The control rats (Group 1) received 0.1 ml of 30% ethanol.

The results showed that EGCG reduced the prostate weight of normal rats by about 30% and the prostate weight of castrated rats injected with testosterone by about 23% during the 7-day study period. There was no reduction of the prostate weight of castrated rats injected with 5α-DHT suggesting that, at least in part, the EGCG effect on the prostate weight loss was due to inhibition of 5α-DHT formation from testosterone.

EGCG also reduced the body weight of normal and castrated rats injected with either testosterone or 5α-DHT by 8 ± 1% during the 7 days period. Unlike the prostate weight loss there was no androgen specificity for the EGCG effect on body weight loss. EGCG appeared to affect the weight loss by a mechanism that reduced fat accumulation. A thorough examination of various organs and blood by a veterinary pathologist did not reveal any abnormal growth or pathogenic effects of EGCG.

5.9.4 Selective Reduction of Fresh Organ Weights and Body Weight by EGCG

Sprague–Dawley male rats, body weight 175 g ± 5 g and 5 rats per group, were injected with 0.3 ml of 30% ethanol containing 10 mg or 15 mg of one of the following catechins obtained from green tea: EC, ECG, EGC, EGCG. The control rats were injected with 0.3 ml of 30% ethanol. With EGCG, the body weight reduction was about 10% at the dose of 10 mg per day and about 25% at 15 mg per day. No significant effect on the body weight was seen with ECG and EGC at up to 15 mg per day (FIG. 21).

EGCG, at 10 mg per day, resulted in about 30% reduction in the weight of ventral prostate (FIG. 19), dorsolateral prostate, and coagulating glands. The weight loss for seminal vesicles and preputial glands was about 20 to 25% (FIG. 20). At 15 mg per day, EGCG reduced the weight for all of these organs by 60% or more. The EGCG effect on the weights of testis and kidney was not significant with EGCG at 10 mg per day and was about 10% or less at 15 mg per day. ECG effect, if any, at 10 and 15 mg per day was less than 20% for all organs examined.

The fact that, at 10 mg per day dose of EGCG, the body weight loss was less than 10% while the prostate weight loss was more than 30% indicated that the prostate weight loss may not be the direct consequence of the body weight loss. However, for both the body weight and the organ weight loss, the structural preference for one –OH group in EGCG that is not in the ECG molecule is very important. The loss in the body weight and the organ weights may be due to EGCG interference of a common step that is required for the body and organ weight gain.

Since it appeared that the loss in the body fat weight may be responsible for the whole body weight loss, EGCG may interact and interfere with a receptor macromolecule (probably containing a protein) that can modulate specific lipid synthesis or accumulation. Lipids can modulate gene expression, cell development and differentiation, and organ growth. Specific interference of the role of lipids in the cells and organs may control the growth of organs, such as, prostate, sebaceous organs, preputial organs, and other secretory organs. Benign or abnormal growth or cancer of these organs can therefore, be chemoprevented or treated by EGCG and related compounds.

These studies demonstrate that the body weight was reduced significantly only by EGCG. Moreover, ECG (containing 7-OH groups) that contains one less -OH group than EGCG (containing 8-OH groups) was not very active or not active at all.

When the EGCG application was stopped, the body weight recovered to about the same weight as for the control animal, indicating that he effect of EGCG was reversible. The results demonstrated that EGCG does not cause a permanent toxicity or damage to the rats (FIG. 22).

Similar effects of EGCG was found with the organ weight for preputial organ, ventral prostate, dorsolateral prostate, coagulating gland, and seminal vesicles. EGCG effect on kidney and testis was not as significant as these androgen-sensitive organs. The EGCG effect could be observed clearly at 10 mg/rat but ECG was much less active at 10 mg/rat. Androgen sensitive male accessory organs (ventral and dorsolateral prostates, seminal vesicles, and other organs were affected to a greater extent than testis of kidney.

5.10 Example 10 -- Prostate and Breast Cancer Growth in Nude Mice

Human prostate cancer PC-3 cells were grown in culture medium. About one million cells were injected into male nude mice and the growth of tumors were followed. Within two weeks, the tumor grew to about 100 mm^3. Three tumor bearing mice were injected with 1 mg EGCG in 0.1 ml water each day. The 3 controls received water only.

Tumors in the EGCG injected mice did not grow during the following two weeks and the size of tumors were smaller than 100 mm^3 (FIG. 17). Tumors in the control mice (not injected with EGCG grew to about 450 mm^3 in two weeks and to about 650 mm^3 in three weeks. When EGCG injection was stopped two weeks later (arrow) tumors in the EGCG injected mice showed new growth.

This was also observed in another study (FIG. 18). The tumor in the control (not injected with EGCG) mouse grew to about 1400 mm^3 within 2 weeks. EGCG was then administered at this time. The tumor size reduced during the next 2 weeks to about 850 mm^3 and was about 500 mm^3 within another 2 weeks.

The tumor in the EGCG injected mouse was about 100 mm^3 during the 2 week injection. When EGCG injection was stopped, the tumor size started to grow to about 800

mm^3 within 2 weeks and was over 1200 mm^3 within 3 weeks. The results showed that EGCG was effective in chemoprevention and chemotherapy of the human prostate cancer. For breast tumor studies, human breast tumor cell line, MDF-7 (1 million cells) was injected into female nude mice. After 5 weeks, the tumor was over 1000 mm^3. EGCG (1 mg/mouse/day) injection for 2 weeks reduced the tumor by 50%).

5.11 Example 11 -- Inhibition of Human Prostate Tumor Growth Using Androgen Compositions

To mimic the natural course of human prostate cancer, the inventors have derived LNCaP 104-R2 cells from the androgen-dependent LNCaP 104-S cells, after long term culture in androgen-depleted medium (Kokontis *et al.*, 1994). LNCaP 104-R2 cells contain AR but their proliferation is not dependent on androgen. Instead, these cells are proliferatively repressed by very low concentrations of androgen in culture medium. It is reported here that testosterone prevents and suppresses the growth of LNCaP 104-R2 tumors in nude mice and that this effect was dependent on the conversion of testosterone to 5 α-DHT.

5.11.1 Materials and Methods

5.11.1.1 Cell Lines

Androgen-dependent LNCaP 104-S (passage 37) and androgen-independent LNCaP 104-R± sublines were isolated as described previously (Kokontis *et al.*, 1994). The characteristics of these cells *in vitro* were confirmed before injection into nude mice. Briefly, proliferation of LNCaP 104-S cells increased 10-13 fold in media containing 0.1 nM of a synthetic androgen, R1881 compared to cells cultured in media depleted of androgen by charcoal-treatment of the fetal bovine sera (FBS) added to the media. LNCaP 104-R2 cells grew in charcoal-treated media without additional androgen. Their proliferation was not stimulated but was repressed by 0.1 nM R1881. LNCaP 104-S cells were maintained in DMEM (Gibco) supplemented with 1 nM 5α-DHT and 10% FBS (Summit Biotechnology) and LNCaP 104-R2 cells were maintained in DMEM supplemented with 10% FBS treated with charcoal to remove steroid (Kokontis *et al.*, 1994). PC-3 and MCF-7 cell lines were

obtained from the American Type Culture Collection (Rockville, MD), and were maintained in DMEM supplemented with 10% FBS.

5.11.1.2 Animals

BALB/c athymic (nude) male (LNCaP, PC-3 cell lines) and female (MCF-7 cell line) mice (Taconic Inc., Germantown, NY), 5 to 7 weeks-old, were used. Mice were housed in a pathogen-free environment, four to five mice per cage. Cages (filter top), bedding and water were autoclaved before use. Feed was irradiated Pico Lab Mouse Chow 20 5058 (Purina). All procedures involving animals were approved by the University of Chicago Institutional Animal Care and Use Committee. For the tumor growth studies, 10^6 cells in 0.25 ml of culture medium were mixed with 0.25 ml of Matrigel™ (Collaborative Research, Bedford, MA) and were injected subcutaneously into one or both flanks of the mice as described previously (Liao *et al.*, 1995). Tumor size was measured weekly and tumor volume was calculated using the formula $L \times W \times H \times 0.52$ (Janek and Hartman, 1975). Bilateral orchiectomy and subcutaneous implantation or removal of pellets were performed under Metofane anesthesia. Blood samples were obtained by heart puncture or from the orbital plexus while mice were under anesthesia and analyzed for testosterone levels by radioimmunoassay or PSA levels by dual-site reactive enzymatic immunoassay (Tandem®-E PSA, Hybritech, San Diego, CA). All steroid hormone (20 mg) pellets were purchased from Hormone Pellet Press (Westwood, KS). Finasteride (Proscar®, 5 mg, Merck, NJ) was obtained from the University of Chicago hospital pharmacy. All numerical data are expressed as the average of the values obtained from 4 to 6 tumors and the standard error.

5.11.1.3 RNA Analysis

Total RNA was isolated from tumor tissue using the acid-guanidium thiocyanate phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Ribonuclease protection assay (Zinn *et al.*, 1983; Hay *et al*, 1987) was performed using probes generated from a 210-bp *KpnI-SacI* fragment of human AR cDNA (Kokontis *et al.*, 1994; Chang *et al.*, 1988) a 77-bp fragment of human PSA cDNA (Kokontis *et al.*, 1994; Young *et al.*, 1991), a 252-bp *PstI-ClaI* fragment of human c-myc cDNA (Alitalo *et al.*, 1983) and a

144-bp *Pst*I-*Hinc*II fragment at the 5' terminus of human β_2-microglobulin (Suggs *et al.*, 1981). Inclusion of a β_2-microglobulin antisense RNA probe in hybridizations served as internal standard for normalization of samples containing different levels of total RNA.

5.11.1.4 Sequencing of LNCaP Androgen Receptor mRNA From Tumors

cDNA encoding LNCaP AR androgen-binding domain was amplified by RT-PCR™ (Kokontis *et al.*, 1991) using the primers 5'-GGCGATCCTTCACCAATGTC-3' (AR nucleotide sequence number 2780-2799) (SEQ ID NO:1) and 5'-GGAAAGGTCCACGCTCACCAT-3' (AR nucleotide sequence number 3184-3203) (SEQ ID NO:2) (Chang *et al.*, 1988). Gle-purified PCR™ products (424 base pairs) were inserted into the *Eco*RV site of pBlueScript SK(+) (Stratagene) and sequenced by a double-stranded DNA dideoxy sequencing method using Sequenase (Amersham).

5.11.1.5 Histology and Immunocytochemistry

For histological examination, resected tumor tissues were fixed in 10% formalin, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin. Immunolocalization studies on paraffin sections used a rabbit polyclonal anti-human AR antibody (AN-15) (5 μg protein/ml) that is directed against amino acids 1 through 15 of the amino-terminus of AR and polyclonal anti-human PSA antibody (15 μg protein/ml) (DAKO, Carpenteria, CA). Nude mice tumors originating from PC-3 cells were used as negative controls. Immunostaining was carried out using a streptavidin-biotin-peroxidase protocol (Liang *et al.*, 1993). For AR immunostaining, deparaffinized tissue sections were pretreated with microwave irradiation in citrate buffer for 5 min (Hobisch *et al.*, 1995).

5.11.1.6 Abbreviations

AR, androgen receptor; TP, testosterone propionate; R1881, 17 β-hydroxy-17 α-methyl-estra-4,9,11-trien-3-one; DHT, dihydrotestosterone; DMEM, Dulbeccos' Modified Eagle medium; FBS, fetal bovine serum; PSA, prostate specific antigen; RT-PCR™ reverse transcriptase polymerase chain reaction; TGF-β, transforming growth factor-β1.

5.11.2 **Results**

5.11.2.1 **Tumorigenicity of LNCaP 104-S and LNCaP 104-R2 Cells in Nude Mice**

Palpable tumors were detected in 83% of normal mice, but 0% of castrated mice (Table 12) weeks after injection of LNCaP 104-S cells. In contrast, 5 weeks after injection of LNCaP-R2 cells, palpable tumors were detected in 75% of castrated mice, but 0% of normal mice. However, 7 weeks after injection, palpable LNCaP 104-R2 tumors were detected in 50% of normal mice and their average size was 831 ± 191 (SE) mm^3, which was almost the same size as tumors found in castrated mice (884 ± 64 (SE) mm^3) at this time. LNCaP cells have a point mutation from A to G (Kokontis *et al.*, 1991; Veldscholte *et al.*, 1990) at nucleotide position 3157 (Chang *et al.*, 1988) in the DNA coding for the androgen-binding domain of AR. It was found that AR cDNA derived from LNCaP 104-S or 104-R2 tumors also have this mutation, which is consistent with these tumors originating from the injected LNCaP cells.

TABLE 12

TUMORIGENICITY OF LNCaP 104-S and LNCaP 104-R IN NUDE MICE[a]

	Tumor Incidence							
	LNCaP 104-S				LNCaP 104-R2			
Week	Normal		Castrated		Normal		Castrated	
	No.	%	No.	%	No.	%	No.	%
3	0	(0)	0	(0)	0	(0)	0	(0)
4	10	(83)	0	(0)	0	(0)	9	(0)
5	10	(83)	0	(0)	1	(0)	9	(75)
7	10	(83)	0	(0)	4	(33)	9	(75)
7	11	(91)	0	(0)	6	(50)	10	(83)

[a]LNCaP cells were injected into 12 normal male nude mice and 12 nude mice castrated 24 hours before cell injection. Mice with palpable tumors were identified every week. No tumors were found three weeks after cancer cell injection. The number of tumor bearing mice is shown under (No.). The percentage of tumor bearing mice is shown in parenthesis.

5.11.2.2 Effect of Androgens and Other Steroid Hormones on the Growth of NCaP 104-R2 Tumors

If a testosterone propionate pellet (TP) was implanted at the 4th week in castrated nude mice with growing LNCaP 104-R2 tumors, further tumor growth was inhibited and tumor size was significantly reduced to about 100 mm^3 or less at the 7th week (FIG. 23). A similar tumor suppressive effect was observed when testosterone or 5α-dihydrotestosterone pellets were implanted. 5β-dihydrotestosterone, a nonandrogenic stereoisomer of 5α-dihydrotestosterone was not effective, suggesting that the suppressive effect required androgenic steroids. 17β-estradiol and medroxyprogesterone acetate were not suppressive and actually showed some growth stimulatory activity.

5.11.2.3 Effects of Testosterone Propionate on the Growth of Other Tumors

In contrast to LNCaP 104-R2 tumors, proliferation of LNCaP 104-S tumors was stimulated by androgens (FIG. 24). If tumor bearing nude mice were castrated 4 weeks after injection of cells, growth of LNCaP 104-S tumors stopped and, during the next 4 weeks, tumors regressed to 10% of their size before castration. If TP was implanted at the time of castration, the tumors continued to grow from 299 ± 27 (SE) mm^3 to 965 ± 166 (SE) mm^3 during the next 4 weeks. TP did not affect the growth of AR negative PC-3 tumors. In female nude mice, the growth of MCF-7 tumors, which express both estrogen and androgen receptors, was also not affected by TP. Therefore, the androgen-dependent suppression of LNCaP 104-R2 tumor growth was both tumor and steroid specific.

5.11.2.4 Androgen-Dependent Remission of LNCaP 104-R2 Tumors and its Reversal by Removal of TP or Implantation of Finasteride

The LNCaP 104-R2 tumors in the control castrates grew to 884 ± 64 (SE) mm^3 in castrated mice 7 weeks after injection of cells (FIG. 25 and FIG. 26A). TP implantation in these mice resulted in a rapid reduction in tumor size. The effect of TP was clearly visible within one week; massive hemorrhage was seen in tumors (FIG. 26B). Four weeks after TP implantation, tumor size was reduced to 208 ± 33 (SE) mm^3 (FIG. 25 and FIG. 26C). If TP was removed at the 7th week from LNCaP 104-R2 tumor bearing mice that were originally implanted with TP at the 4th week (FIG. 23), tumors regrew from 96 ± 26 (SE) mm^3 (FIG. 25 and FIG. 26D) to 641 ± 157 (SE) mm^3 (FIG. 25 and FIG. 26E) within the next 4 weeks.

5-AR inhibitors (Russell and Wilson, 1994), such as finasteride can present testosterone action that is dependent on the conversion of testosterone to 5α-DHT (Bruchosky and Wilson, 1968; Anderson and Liao, 1968). Therefore, the inventors studied whether finasteride can prevent the TP-dependent suppression of LNCaP 104-R2 tumors in nude mice. When finasteride (2.5 mg) pellets were implanted at the 7th week in mice originally implanted with TP at the 4th week, LNCaP 104-R2 tumor growth resumed from the TP repressed level of 84 ± 15 (SE) mm^3 and reached 593 ± 144 (SE) mm^3 within 4 weeks (FIG. 25 and FIG. 26F). The rate of this regrowth was about the same as that in nude mice from which implanted TP was removed (FIG. 25 and FIG. 26F). Thus, finasteride alleviated the testosterone suppression of tumor growth.

In contrast, finasteride treatment of LNCaP 104-S tumors, in normal nude mice, reduced tumor size by 45%, from 1,387 ± 432 (SE) mm^3 to 759 ± 136 (SE) mm^3 within 4 weeks (FIG. 27). During this period, the tumor size in the control mice without finasteride implant increased by 240%. Thus, 5α-DHT played a major role in maintaining the growth of LNCaP 104-S tumors. Finasteride did not affect the growth of human breast MCF-7 tumors in female nude mice.

5.11.2.5 Histology

There was no clear histological difference between LNCaP 104-R2 and LNCaP 104-S tumors grown in nude mice. For LNCaP 104-R2 tumors, no remarkable histological change was noted within 3 days after TP implantation (FIG. 28A). At 5-7 days after TP implantation, histological sections revealed extensive necrosis with severe hemorrhage (FIG. 28B). At the 4th week after TP treatment, tumor size was markedly decreased, and histological sections revealed fibrosis with infiltration of chronic inflammatory cells and scattered carcinoma cells in the process of degeneration (FIG. 28C).

5.11.2.6 Effect of Androgen on the Expression of Androgen Receptor, c-*myc*, and PSA by LNCaP 104-R2 Tumors

Immunocytochemical staining of LNCaP 104-R2 tumors localized AR to the nucleus (FIG. 28D) and PSA to the cytoplasm (FIG. 28E) in tumor cells but not in surrounding mouse cells. The level of mRNA for AR and c-*myc* in the LNCaP 104-R2 tumor was reduced by about 50 to 70% within 3 days after TP implantation (FIG. 29). This initial rapid loss preceded the general loss of tumor cells. The level of PSA mRNA in tumor samples (FIG. 29) and serum PSA increased more than 10-fold after 1 week of TP treatment and remained at this high level for at least one more week. At this early stage of TP action, enhanced PSA expression indicates that some tumor cells are viable and still respond to androgenic stimulation.

5.11.2.7 Biological Effects of Androgen in Nude Mice

The results suggest that TP implants were biologically effective for at least 7 weeks. TP used in the studies maintained the serum testosterone level at 20 to 28 ng/ml for at least 7 weeks. In comparison, the serum testosterone level was about 5 ng/ml in normal and 0.3 ng/ml in castrated male mice without TP implants. Since TP stimulated the growth of tumors derived from LNCaP 104-S cells and had no effect on the growth of PC-3 and MCF-7 tumors in nude mice, it is unlikely that the growth suppression of LNCaP 104-R2 tumor by TP was due to a general toxicity of implanted androgen. This conclusion is supported by the fact that at the 4th week after androgen implantation, the seminal

vesicle weight in the nude mice with either LNCaP 104-S or 104-R2 tumors increased about 10 times (compared to that in castrates without TP treatment) and there was no loss in the body weight of these nude mice.

5.11.3 Discussion

Androgens are necessary for normal prostate development and function. Most newly diagnosed prostate cancers are also androgen dependent. However, the human prostate cancer cells lines, LNCaP 104-R1 (Liao *et al.*, 1995) and 104-R2 cells±, which contain a very high level of AR (over 10-fold more than the androgen stimulatory LNCaP 104-S cells), are not proliferatively stimulated by androgen but are actually repressed by low concentrations (0.1 nM) of androgens. It has been reported that the proliferation of PC-3 cells transfected with an AR expression vector also is inhibited by androgen in culture (Yuan *et al.*, 1993). It was found that PC-3 cells retrovirally infected with an AR expression vector do not survive well in culture.

Since androgens inhibited the growth of LNCaP 104-R cells in culture (Kokontis *et al.*, 1994), androgen may exert its effect directly on the tumor cells in nude mice. Excessive expression of androgen-induced gene(s) may result in an imbalance in coordination of various cellular functions or a change in the production of factors that affect cell cycling or apoptosis. For example, TGF-β1 mRNA level in the rat ventral prostate is negatively controlled by androgen (Kyprianou and Issacs, 1989), whereas inhibition of LNCaP cell proliferation by TGF-β1 in culture (Wilding, 1991) is dependent on the presence of an appropriate concentration of androgen (Kim *et al.*, 1996). Androgen also suppresses the expression of prostatic sulfated glycoprotein-2 (Clusterin) (Bettuzzi *et al.*, 1989; Monpetit *et al.*, 1986), which prevents LNCaP cell death induced by tumor necrosis factor α (Sensibar *et al.*, 1995). Tumor growth is dependent on tumor angiogenesis (Weidner *et al.*, 1993). However, histological analysis did not reveal a clear effect of testosterone on vascularization in the LNCaP 104-R2 tumor during the initial weeks of tumor growth suppression.

Androgen-repressed LNCaP 104-R2 tumors slowly adapted to growth in the presence of androgens. In normal male mice, LNCaP 104-R2 cells did not grow into palpable tumors in 4 weeks. However, in 50% of these mice, they slowly adapted to the

presence of androgen over a 7 week period and grew to a size equivalent to LNCaP 104-R2 cells grown in castrated nude mice for 7 weeks (Table 12). It has been suggested that intermittent use of androgen may delay prostate cancer cell progression (Goldenberg *et al.*, 1995). These observations indicated that some prostate tumors that would be considered androgen-independent may revert to an androgen-sensitive phenotype. These tumors may then be responsive to androgen-ablation therapy.

The derivation of LNCaP 140-R2 cells from LNCaP 104-S cells after a long period (2 years) of culture in androgen-depleted culture medium may mimic the situation in prostate cancer patients who receive androgen ablation therapy (orchiectomy or chemical castration) (Dawson and Vogelzang, 1994; Coffey, 1993; Geller, 1993). Prostatic tumors in these patients initially respond to androgen ablation therapy, but prostate cancer often reappears as an androgen-independent cancer. A recent report showed that distant metastases in patients with prostatic carcinoma who have undergone various kinds of endocrine therapy contain AR (Hobisch *et al.*, 1995). Some of these metastatic prostate tumor cells may behave like LNCaP 104-R2 cells and respond to androgen-suppression or revert to androgen-dependent tumors as shown in the present study.

The 5-AR inhibitor, finasteride, has been found to be effective in the treatment of benign prostatic hyperplasia in some patients (Stoner and Finasteride Study Group, 1994). Finasteride is also being tested for the chemoprevention of prostate cancer (Gormley *et al.*, 1995). The present findings indicate that testosterone-suppression of LNCaP 104-R2 tumor growth required conversion of testosterone to 5α-DHT and that finasteride reversed this suppressive effect and promoted the regrowth of LNCaP 104-R2 tumors. It is, therefore, important to consider this adverse effect, if finasteride is to be used in prostate cancer chemotherapy. Flutamide (an antiandrogen being used for prostate cancer therapy) stimulates the growth of LNCaP cells (Wilding *et al.*, 1989) because the AR in these cells has a point mutation in the ligand-binding domain and can utilize antiandrogenic hydroxyflutamide as an androgen to transactivate target genes (Kokontis *et al.*, 1991; Veldscholte *et al.*, 1990). Effective use of antiandrogens and 5-AR inhibitors for prostate cancer therapy, therefore, needs careful assessment of the particular type of prostate cancer cells present.

LNCaP 104-R (Kokontis *et al.*, 1994) is now designated as LNCaP 104-R1. LNCaP 104-R1 cells were derived from androgen-dependent LNCaP 104S cells after 40 passages in DMEM containing charcoal-stripped FBS, whereas LNCaP 104-R2 cells were derived from LNCaP 104-R1 cells after 60 additional passages in the same androgen-depleted medium.

6. REFERENCES

The references listed below and all references cited herein are incorporated herein by reference to the extent that they supplement, explain, provide a background for, or teach methodology, techniques, and/or compositions employed herein.

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* * * * * * * * * * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Arch Development Corporation

(B) STREET: 1101 East 58th Street

(C) CITY: Chicago

(D) STATE: Illinois

(E) COUNTRY: United States of America

(F) POSTAL CODE (ZIP): 60637

(ii) TITLE OF INVENTION: Methods and Compositions for Inhibiting 5α-Reductase Activity

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/442,055

(B) FILING DATE: 16-MAY-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGCGATCCTT CACCAATGTC 20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGAAAGGTCC ACGCTCACCA T 21

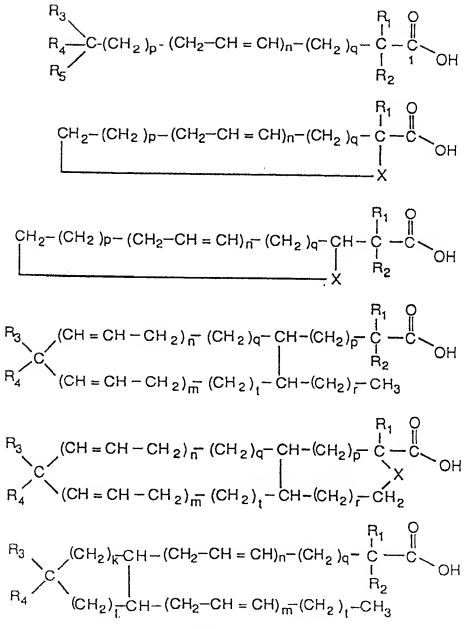
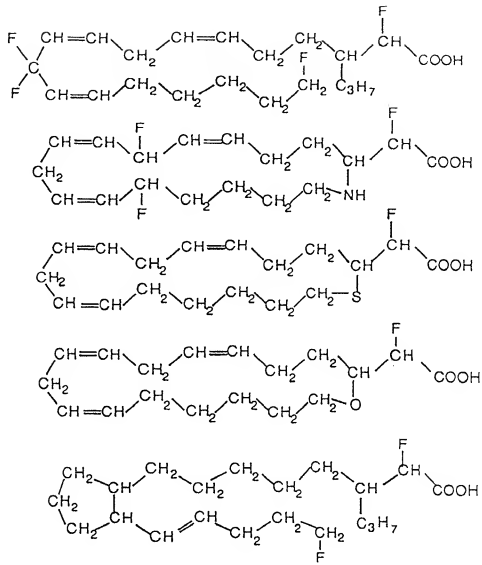


FIG. 1

$CH_3(CH_2)_nCH(F)COOH$ (n=0~17)	[Gershon & Parmegliani, J.Med.Chem.10:186(1967)]
$F(CH_2)_nCOOH$ (n=1~19)	[Pattison et al.J.Org. Chem.21:883(1956)]
$FCH_2(CH_2)_{7-n}CH=CH(CH_2)_{7+n}COOH$ (n=0~7)	[Peters & Hall, Biochem. Pharmacol.2:25(1959)]
$F(CH_2)_nCH(R)(CH_2)_mCOOH$ (n=5~8;m=0~8;R=CH_3,C_2H_5,C_3H_7)	[Pattison & Peters, Biochem.J.98:680(1966); Pattison & Buchanan, Biochem.J.92:100(1964)]
$CF_3(CH_2)_{14}CF_2CF_2COOH$	[Stoll et al., J.Lipid Res.32:843(1991)]
$CH_3(CH_2)_{13-m}CF_2(CH2)_{m-2}COOH$ (m=0~13)	[Gent & Ho, Biochemistry 17:3023(1978)]

FIG. 2



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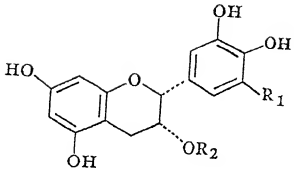

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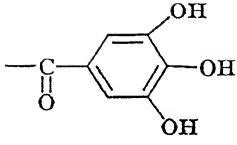

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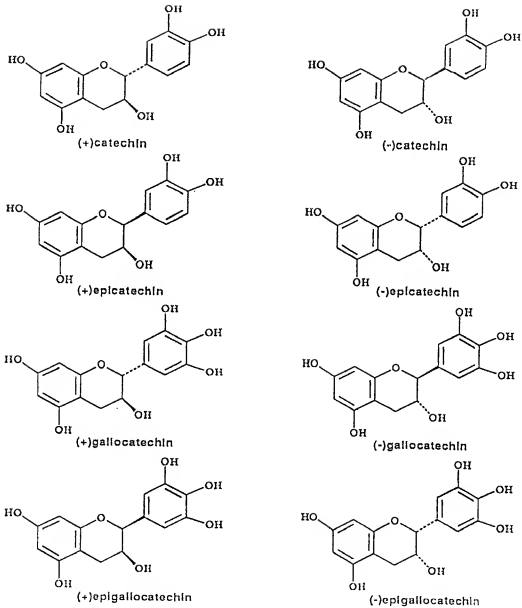

(+)catechin
(-)catechin
(+)epicatechin
(-)epicatechin
(+)gallocatechin
(-)gallocatechin
(+)epigallocatechin
(-)epigallocatechin

FIG. 6

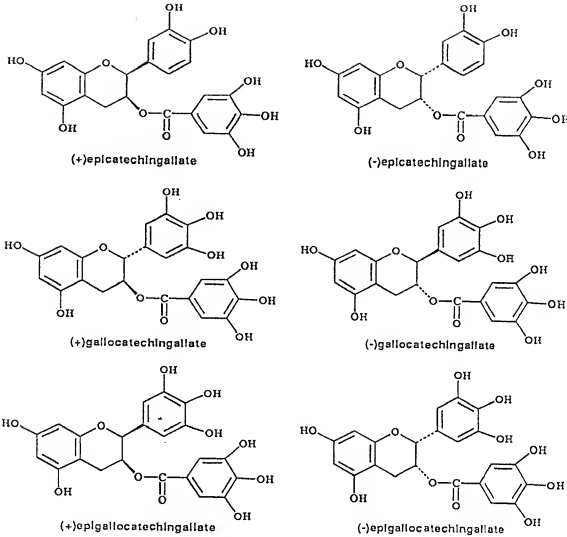

(+)epicatechingallate
(-)epicatechingallate
(+)gallocatechingallate
(-)gallocatechingallate
(+)epigallocatechingallate
(-)epigallocatechingallate

FIG. 7

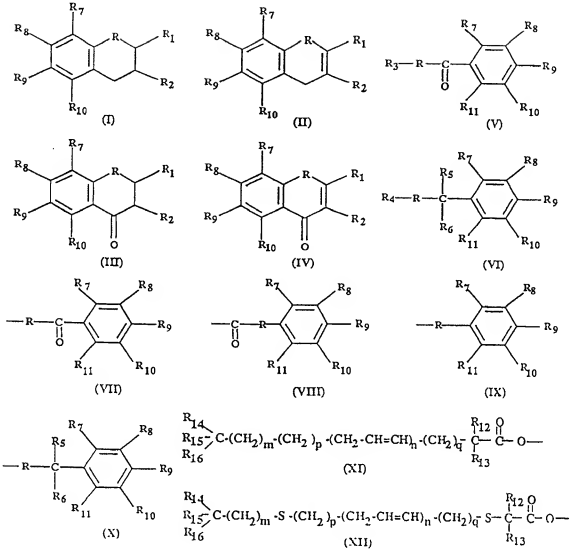
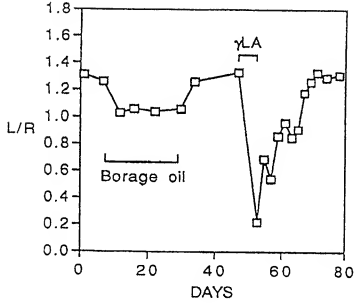


FIG. 8



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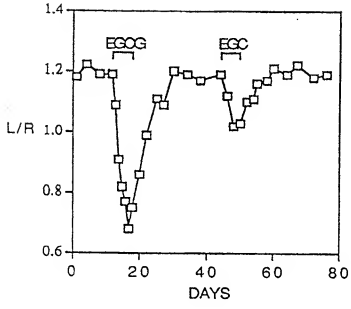
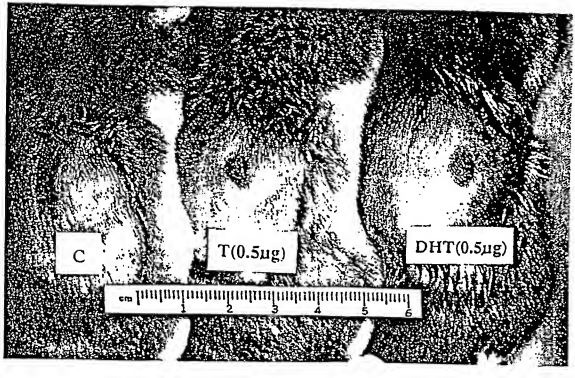


FIG. 10



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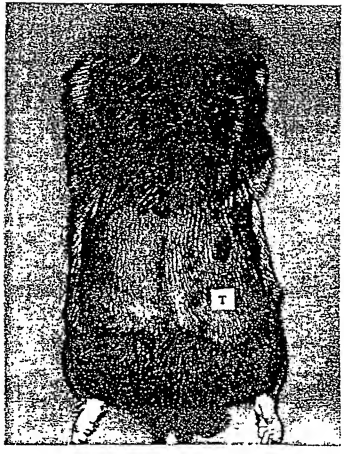
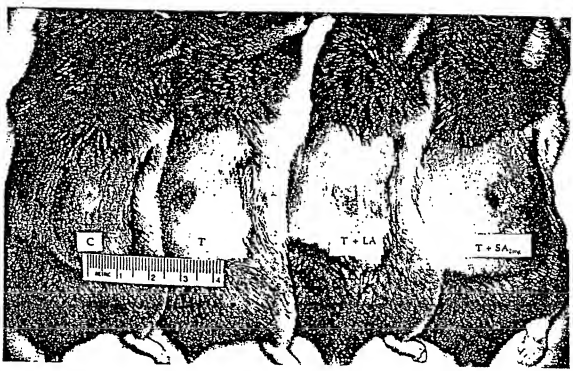


FIG. 12



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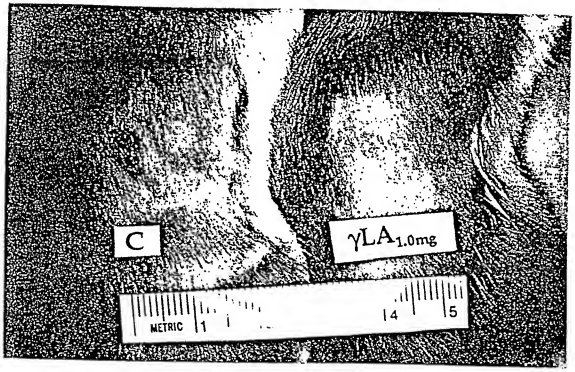
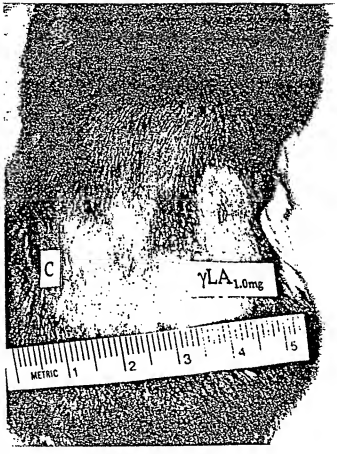


FIG. 14



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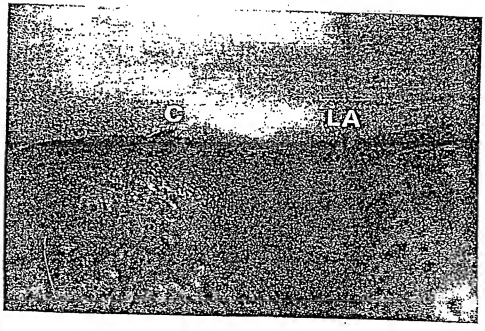

FIG. 16

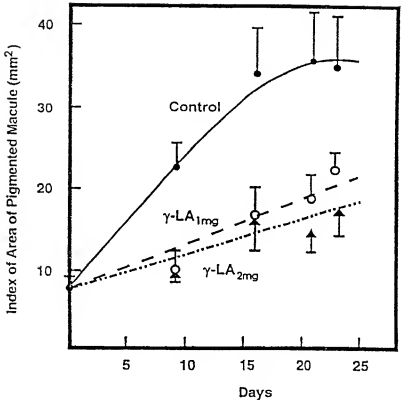
Index of Area of Pigmented Macule (mm²)
40
30
20
10
Control
γ-LA1mg
γ-LA2mg
5
10
15
20
25
Days

FIG. 17

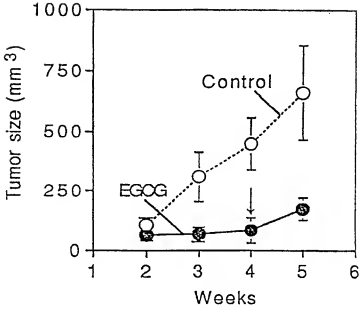

FIG. 18

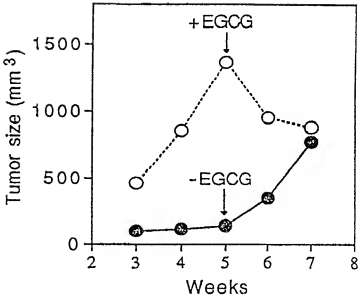

FIG. 19

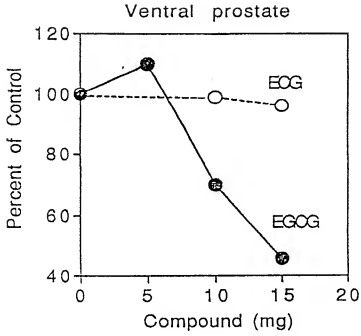
Ventral prostate
120
100
80
60
40
Percent of Control
EOG
EGCG
0
5
10
15
20
Compound (mg)

FIG. 20

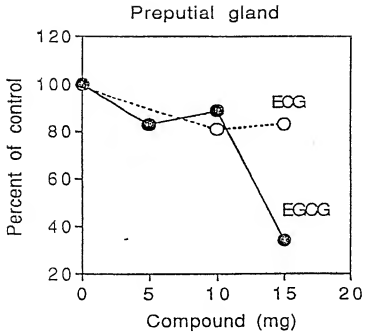
Preputial gland
120
100
80
60
40
20
Percent of control
0
5
10
15
20
Compound (mg)
ECG
EGCG

FIG. 21

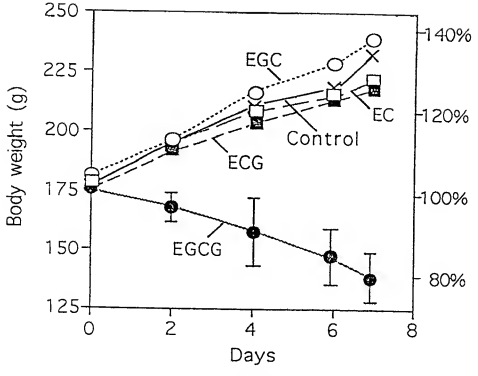
250
225
200
175
150
125
Body weight (g)
0
2
4
6
8
Days
140%
120%
100%
80%
EGC
EC
Control
ECG
EGCG

FIG. 22

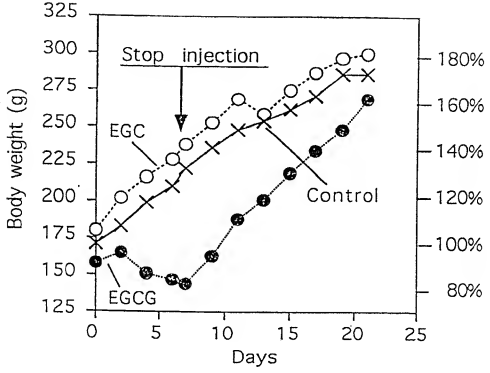
325
300
275
250
225
200
175
150
125
Body weight (g)
Stop injection
EGC
Control
EGCG
180%
160%
140%
120%
100%
80%
0
5
10
15
20
25
Days

FIG. 23

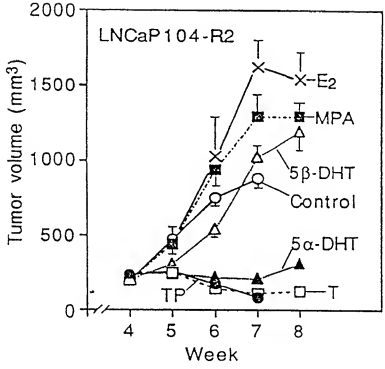
LNCaP104-R2
Tumor volume (mm3)
2000
1500
1000
500
0
4
5
6
7
8
Week
E2
MPA
5β-DHT
Control
5α-DHT
T
TP

FIG. 24

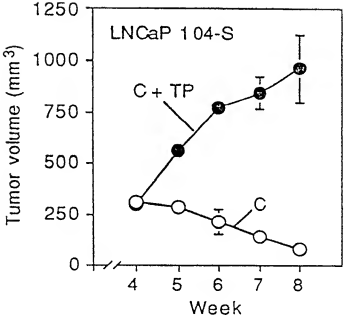
LNCaP 104-S
C + TP
C
Tumor volume (mm3)
1250
1000
750
500
250
0
4
5
6
7
8
Week

FIG. 25

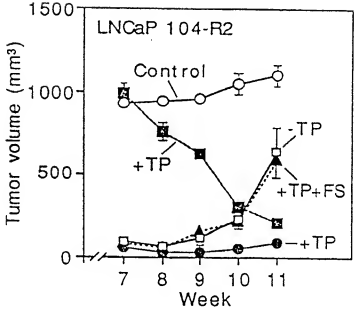
LNCaP 104-R2
Control
-TP
+TP
+TP+FS
-+TP
Tumor volume (mm³)
1500
1000
500
0
7
8
9
10
11
Week

FIG. 26

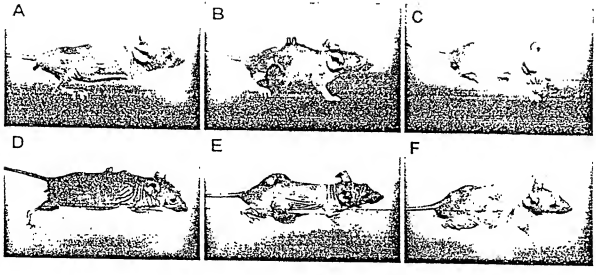

FIG. 27

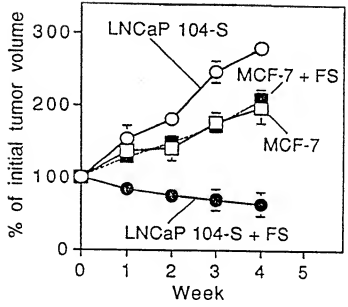
% of initial tumor volume
300
200
100
0
LNCaP 104-S
MCF-7 + FS
MCF-7
LNCaP 104-S + FS
0
1
2
3
4
5
Week

FIG. 28

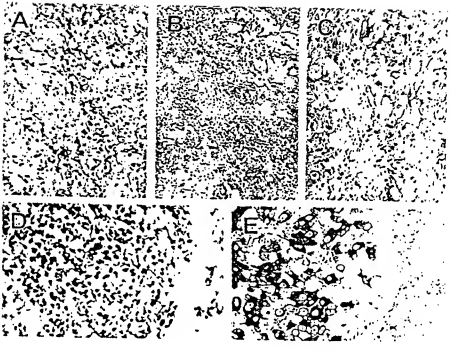

FIG. 29

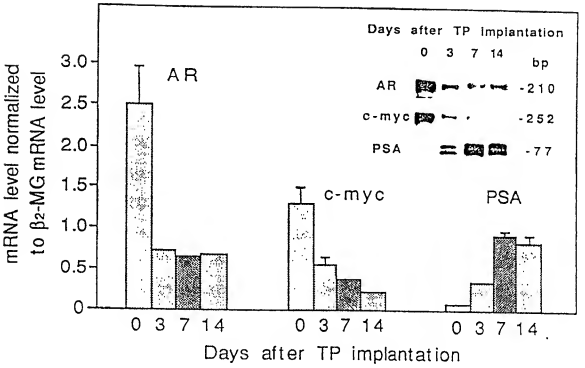
Days after TP implantation
0 3 7 14
bp
AR
-210
c-myc
-252
PSA
-77
AR
c-myc
PSA
mRNA level normalized to β2-MG mRNA level
3.0
2.5
2.0
1.5
1.0
0.5
0
0 3 7 14
0 3 7 14
0 3 7 14
Days after TP implantation

FIG. 30